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# **Plant Hormone Signaling**

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# Contents

<b>Contributors</b>	<b>xiii</b>
<b>Preface</b>	<b>xv</b>
<b>1 Absciscic acid synthesis, metabolism and signal transduction</b>	<b>1</b>
ANNIE MARION-POLL and JEFFREY LEUNG	
1.1 Introduction	1
1.2 Biosynthesis and catabolism pathways	2
1.2.1 Main early steps of ABA biosynthesis	2
1.2.2 Epoxy-carotenoid cleavage	4
1.2.3 The conversion of xanthoxin to ABA	6
1.2.4 ABA catabolism	6
1.3 Regulation of ABA synthesis and metabolism	7
1.3.1 Developmental regulation	7
1.3.1.1 Vegetative tissues	7
1.3.1.2 Reproductive organs	8
1.3.2 Regulation in response to abiotic stresses	9
1.3.3 Regulation by endogenous signals and factors	10
1.4 ABA signaling in seed maturation processes: proteolysis and combinatorial protein interactions	12
1.5 Stress responses in vegetative tissues: the five major nexuses	15
1.5.1 ABA recognition sites and the search for the receptors	15
1.5.2 Transcriptional network as the readout	17
1.5.3 RNA metabolism	17
1.5.4 Protein phosphatases 2C	19
1.5.5 Sucrose non-fermenting-related kinases	19
1.6 ABA signaling in guard cells: simple movements controlled by complex mechanisms	22
1.7 ABA as antagonizing signal to light in stomatal movement	23
1.8 Concluding remarks	24
Acknowledgements	25
References	26
<b>2 Auxin metabolism and signaling</b>	<b>37</b>
JERRY D. COHEN and WILLIAM M. GRAY	
2.1 Introduction	37
2.2 Auxin metabolism	37
2.2.1 Indole-3-acetic acid biosynthesis	37
2.2.1.1 The tryptophan-independent pathway	37

2.2.1.2	IAA biosynthesis from tryptophan	40
2.2.2	IAA conjugates in plants	42
2.2.2.1	IAA-peptide conjugates	42
2.2.2.2	Amino acid conjugates	43
2.2.2.3	Amide conjugate hydrolysis	43
2.2.2.4	Ester conjugates	44
2.2.3	IAA degradation	46
2.3	Auxin signaling	46
2.3.1	Auxin-responsive genes	46
2.3.2	Auxin response factors	47
2.3.3	Regulation of auxin response by the SCF <sup>TIR1</sup> ubiquitin–ligase	51
2.3.4	Regulation of SCF <sup>TIR1</sup> activity	53
2.3.5	Identification of an auxin receptor	55
2.4	Conclusions and future perspectives	58
	Acknowledgements	59
	References	59
<b>3</b>	<b>Integration of brassinosteroid biosynthesis and signaling</b>	<b>67</b>
	MIKLOS SZEKERES and GERARD J. BISHOP	
3.1	Introduction	67
3.2	Metabolism	67
3.2.1	Biosynthesis	67
3.2.1.1	<i>DET2</i>	69
3.2.1.2	<i>SAX1</i>	72
3.2.1.3	<i>DWF4</i>	72
3.2.1.4	<i>CPD</i>	73
3.2.1.5	<i>ROT3</i> and <i>CYP90D1</i>	73
3.2.1.6	<i>CYP85A1</i> and <i>CYP85A2</i>	74
3.2.1.7	Other biosynthetic functions	74
3.2.2	Inactivation	75
3.2.2.1	<i>BAS1</i>	75
3.2.2.2	<i>CHI2/SHK1/SOB7</i>	76
3.2.2.3	<i>UGT73C5</i>	77
3.2.2.4	<i>BNST3</i> and <i>BNST4</i>	77
3.2.3	Functional aspects of BR metabolism	77
3.2.3.1	Regulation of biosynthetic genes	77
3.2.3.2	Regulation of BR-inactivating genes	78
3.2.3.3	Conservation of BR synthesis in higher plants	79
3.3	Signal transduction	80
3.3.1	<i>BRI1</i> and <i>BAK1</i>	80
3.3.2	<i>BIN2</i> and <i>BSU1</i>	81
3.3.3	<i>BZR1</i> and <i>BZR2/BES1</i>	82
3.3.4	<i>BIM1</i>	82
3.3.5	Signaling mechanism and other putative components	83
3.4	Future perspectives	84
3.4.1	Metabolism	84
3.4.2	Signal transduction	86

3.4.3 Crops	86
Acknowledgements	87
References	87
<b>4 Cytokinin metabolism and signal transduction</b>	<b>93</b>
ALEXANDER HEYL, TOMÁŠ WERNER and THOMAS SCHMÜLLING	
4.1 Introduction	93
4.2 Cytokinin metabolism	93
4.2.1 Cytokinin biosynthesis	94
4.2.2 Cytokinin interconversion and conjugation	97
4.2.3 Cytokinin catabolism	98
4.3 Cytokinin signal transduction	101
4.3.1 Cytokinin signal perception	101
4.3.2 Cytokinin signal transduction	105
4.4 Conclusions	117
References	118
<b>5 Ethylene biosynthesis and signaling: a puzzle yet to be completed</b>	<b>125</b>
FILIP VANDENBUSSCHE, WIM H. VRIEZEN and DOMINIQUE VAN DER STRAETEN	
5.1 Introduction	125
5.2 Ethylene biosynthesis	126
5.2.1 ACC synthase	127
5.2.2 ACC oxidase	130
5.3 Ethylene signal transduction	131
5.4 A complex network	137
Acknowledgements	139
References	139
<b>6 Gibberellin metabolism and signal transduction</b>	<b>147</b>
STEPHEN G. THOMAS and PETER HEDDEN	
6.1 Introduction	147
6.2 The gibberellin metabolic pathway	148
6.2.1 Biosynthesis of bioactive GAs	148
6.2.2 GA deactivation	150
6.3 Genes of GA biosynthesis and their regulation	151
6.3.1 Developmental regulation	151
6.3.2 Hormonal regulation	154
6.3.3 Environmental regulation	154
6.4 The gibberellin signal transduction pathway	157
6.4.1 The gibberellin receptor	159
6.4.2 DELLA proteins act as repressors of GA signaling	159

6.4.3	GAs promote rapid degradation of DELLA proteins	161
6.4.4	SCF <sup>SLY/GID</sup> -mediated degradation of DELLA proteins	162
6.4.5	The role of GID1 in DELLA degradation	164
6.4.6	Additional GA-signaling components	164
6.4.6.1	A role for O-linked N-acetylglucosamine transferases in GA signaling	164
6.4.6.2	DWARF1 and PHOR1, possible positive regulators of GA signaling	165
6.5	Downstream transcriptional events induced by GAs	166
6.5.1	GAMYBs	167
6.5.2	Homoeostatic regulation of GA metabolism	169
6.6	Sites of GA signaling	170
6.6.1	Germinating seeds	170
6.6.2	Stems	171
6.6.3	Flower initiation and development	171
6.6.4	The <i>Arabidopsis</i> root	172
6.7	Conclusions	174
	Acknowledgements	176
	References	176
<b>7</b>	<b>Oxylipins: biosynthesis, signal transduction and action</b>	<b>185</b>
	CLAUS WASTERNAK	
7.1	Introduction	185
7.2	$\alpha$ -Dioxygenase, phytoprostanes and electrophile compounds	187
7.2.1	$\alpha$ -Dioxygenase	187
7.2.2	Phytoprostanes and electrophile compounds	187
7.3	The LOX pathway	189
7.3.1	The LOX	190
7.3.2	HPOT/HPOD: the branch point in the LOX pathway	191
7.3.3	The AOS branch: jasmonate biosynthesis	192
7.3.3.1	The AOS	192
7.3.3.2	The allene oxide cyclase	193
7.3.3.3	OPR3	194
7.3.3.4	$\beta$ -oxidation in JA biosynthesis	194
7.3.3.5	Jasmonate metabolites	197
7.4	Mutants in JA biosynthesis and in JA signaling	199
7.4.1	Mutants in JA biosynthesis	199
7.4.2	Mutants in JA signaling	201
7.4.3	Proteasome-mediated JA signaling	203
7.5	JA, OPDA and related compounds in plant-defense reactions	205
7.5.1	Plant-microbe interactions	205
7.5.1.1	Symbiotic interactions	205
7.5.1.2	Plant pathogen interactions	206
7.5.1.3	Cross-talk between JA, SA, ethylene and ABA	207
7.5.2	The wound-response pathway	208
7.5.3	Direct and indirect defense	211



7.6	JA in development	213
7.6.1	Seedling development and root growth	213
7.6.2	Tuber formation	214
7.6.3	Flower formation	214
7.6.4	Senescence	215
7.7	Concluding remarks	216
	Acknowledgements	217
	References	217
<b>8</b>	<b>Salicylic acid</b>	<b>229</b>
	CHRISTOPHE GARCION and JEAN-PIERRE MÉTRAUX	
8.1	Introduction	229
8.2	Biosynthesis and metabolism of SA	230
8.2.1	SA biosynthesis via the phenylpropanoid pathway	230
8.2.2	SA biosynthesis through the isochorismate pathway	233
8.2.3	Relative contribution of the isochorismate and BA pathway	234
8.2.4	Regulation and localization of SA biosynthesis	234
8.2.5	Metabolism of SA	235
8.2.6	Biosynthesis of MeSA	236
8.3	Signal transduction and mode of action	237
8.3.1	SA-binding sites	238
8.3.2	SA and signal transduction mediated by MAP kinases	239
8.3.3	SA and the central role of NPR1	240
8.3.4	SA and other regulatory proteins	243
8.3.5	SA and the mobile signal	243
8.3.6	SA and global gene expression	244
8.3.7	SA and virus resistance	246
8.4	Conclusions	247
	References	257
<b>9</b>	<b>Hormone distribution and transport</b>	<b>257</b>
	JOHN J. ROSS, GREGORY M. SYMONS, LINDY ABAS, JAMES B. REID and CHRISTIAN LUSCHNIG	
9.1	Concepts and definitions	257
9.2	Auxins: distribution and transport	257
9.2.1	Auxin distribution: old views and new developments	257
9.2.2	Auxin biosynthesis: not restricted to the shoot anymore	258
9.3	Auxin transport	260
9.3.1	Mass-flow-dependent distribution of auxin	260
9.3.2	Polar auxin transport	260
9.3.2.1	Physiological aspects	260
9.3.2.2	Auxin transporters	263
9.3.2.3	Regulation of the carriers	265
9.3.3	Conclusion: a joint effort required for auxin transport?	269
9.4	GAs: distribution and transport	269
9.4.1	Seeds and fruits	270

9.4.2	Vegetative tissues	270
9.4.2.1	Grafting studies	270
9.4.2.2	Can mature shoot tissue synthesise GAs?	272
9.4.2.3	Monocotyledonous species	274
9.4.3	Conclusion: some GAs can undergo long-distance transport, at least in some circumstances	277
9.5	BRs: distribution and transport	277
9.5.1	BR distribution	278
9.5.2	BR transport	278
9.5.2.1	Exogenous BRs	278
9.5.2.2	Endogenous BRs: grafting studies	279
9.5.2.3	BR transport within the shoot?	281
9.5.2.4	“Short-distance” BR transport?	282
9.5.3	Conclusion: endogenous BRs do not undergo long-distance transport	283
9.6	General discussion	283
	Acknowledgements	284
	References	284
<b>10</b>	<b>Reproductive development</b>	<b>293</b>
	MIGUEL A. BLÁZQUEZ and JOSÉ LEÓN	
10.1	Introduction	293
10.2	Flowering time	293
10.2.1	Gibberellins	294
10.2.2	Brassinosteroids	295
10.2.3	Auxins, cytokinins and ethylene	295
10.2.4	Abscisic acid	296
10.2.5	Salicylic acid and the stress-activated transition to flowering	296
10.3	Flower development	297
10.4	Early fruit development	299
10.4.1	Gibberellins	299
10.4.2	Auxin	300
10.4.3	Polyamines	301
10.5	Fruit maturation	302
10.5.1	Ethylene	302
10.5.2	Auxin	303
10.5.3	BRs and ABA	303
10.5.4	Salicylic acid	304
10.6	Conclusions	304
	References	304
<b>11</b>	<b>Seed development and germination</b>	<b>311</b>
	SHINJIRO YAMAGUCHI and EIJI NAMBARA	
11.1	Introduction	311
11.2	Hormonal control of seed development	311
11.2.1	Developmental and physiological phases in seed development	312
11.2.2	Developmental regulators for seed development	313

11.2.3	Regulators of ABA responses in the seed	316
11.2.4	ABA and GA metabolism genes during seed development	317
11.2.5	Regulation of balancing ABA and GA levels during seed development	318
11.2.6	Regulation of ABA and GA action during seed development	319
11.3	Hormonal control of seed germination and post-germinative growth	319
11.3.1	Regulation of GA levels in imbibed seeds	319
11.3.1.1	Light-regulation of GA biosynthesis	320
11.3.1.2	Temperature-regulation of GA biosynthesis	321
11.3.1.3	GA response components in germinating seeds	322
11.3.2	Regulation of ABA levels in imbibed seeds	323
11.3.2.1	<i>De novo</i> ABA biosynthesis and catabolism are involved in regulation of ABA levels	323
11.3.2.2	Light, high temperature, and GA regulation of ABA metabolism	323
11.3.3	Sites of GA biosynthesis and response in imbibed seeds	324
11.3.4	GA and ABA action in the cereal aleurone	327
11.3.4.1	GA and ABA perception	327
11.3.4.2	Crosstalk between GA and ABA action	328
11.3.5	Other hormones: actions of ethylene and brassinosteroids during seed germination	329
11.3.5.1	Ethylene	329
11.3.5.2	Brassinosteroids	330
11.4	Conclusions and perspectives	331
	References	331
	<b>Index</b>	<b>339</b>

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## Preface

In the 18th and 19th centuries experiments on bark ringing and tropisms indicated that plants contain a mobile signal, which was subsequently revealed to be the auxin indole-3-acetic acid (IAA). The first half of the last century saw the chemical identification of many of the major plant signaling molecules (hormones) and although progress in understanding their formation and mode of action was initially slow, recent advances in this field have been spectacular, including the identification of receptors for several hormone classes, with those for abscisic acid, IAA and gibberellin being identified within the last year (2005–2006) (see Chapters 1, 2 and 6, respectively). This would therefore appear to be an ideal time to highlight the recent progress in this field. This preface traces briefly the history of plant hormone discovery and identification, which occurred when methods of analysis were much less sensitive than those available today.

Although auxin was the first plant hormone to be recognised as such, when in 1926 Frits Went demonstrated the presence of a diffusible, growth-promoting substance in oat coleoptiles, the profound effects of ethylene on plant development had been observed and utilised well before this time. As related in Chapter 5, the induction of fruit ripening by ethylene has been unknowingly exploited since ancient times, through, for example, scratching the surface of fruit to induce wound ethylene, or by exposure to smoke, which contains ethylene. The accidental discovery in the Azores in 1874 that smoke from burning fields synchronised flowering in pineapples has been exploited in pineapple production ever since, the smoke being replaced by acetylene and later the ethylene-releasing compound ethephon or naphthalene acetic acid, which induces ethylene production, once the active ingredient had been identified. In 1901 Dimitry Neljubow showed that ethylene was the active component of coal gas that induced diageotropism in dark-grown pea seedlings. It might therefore be claimed that ethylene was the first plant hormone to be discovered, although evidence that plants actually produce this gas was not provided until 1934 and it took many more years for it to be accepted as a *bona fide* plant hormone. It may have been difficult to acknowledge that a gas could function as a hormone, although with the discovery of nitric oxide as a ubiquitous signaling molecule, this concept is now well accepted.

Following Went's discovery of auxin (initially referred to as growth accelerating substance and then as Wuchsstoff, the term auxin was introduced by Kögl in 1932) it took several years and some false trails (see Wildman, 1997 for a fascinating account of the early attempts to define the chemical identity of auxin) before IAA was identified as the active principal present in human urine, which was known to contain IAA already in 1885. IAA was also found to be responsible for the auxin activity present in extracts of yeast and *Rhizopus suinus*, and it was finally identified from a plant source, maize meal, in 1942.

It was a common theme that plant hormones were initially identified from non-plant sources, since their concentrations in plants are generally too low for them to have been chemically detectable from this source by the methods available at the time. Thus, the next class of hormones to be discovered, the gibberellins, were found as growth active secretions of the fungus *Gibberella fujikuroi*. Although crystalline substances, named gibberellins by Teijiro Yabuta, were obtained in the 1930s (see Chapter 6), the first chemical structure, that of GA<sub>3</sub>, was not proposed until 1956. The ability of GA<sub>3</sub> and plant extracts to restore the growth of certain dwarf mutants and induce bolting in rosette plants led several groups to propose that GAs were endogenous plant growth regulators (see review by Phinney, 1983). Their presence in plants was confirmed by the identification of GA<sub>1</sub> in seeds of runner bean in 1958.

The fourth hormone class, the cytokinins, were also first identified from a non-plant source. Several plant and non-plant materials, including coconut milk and yeast extract, were found to stimulate division of plant cells in culture, with autoclaved herring sperm being particularly effective. The active ingredient from herring sperm, named kinetin, was identified in 1955 by Miller *et al.* (1955) as N<sup>6</sup>-furfuryladenine, a rearrangement product of 2-deoxyribosyladenine. The first naturally occurring cytokinin was identified from maize in the early 1960s independently by Letham and Miller, who agreed on the name zeatin (Letham & Miller, 1965). The term cytokinin for this class of hormones was introduced by Skoog *et al.* (1965). Recent advances in cytokinin signaling is described in Chapter 4.

The last of the so-called classical plant hormones, abscisic acid (see Chapter 1), was identified around the same time as zeatin, atypically directly from plant sources, although fungi are also known to produce this compound. Investigations of growth inhibiting substances were carried out in the 1950s and early 1960s, including the purification of compounds that apparently induced abscission of cotton fruit in the laboratory of Frederick Addicot, who named them abscisins, and of a substance (dormin) that induced bud dormancy in trees, by Wareing and co-workers. In 1965, Addicot's group reported the structure of Abscisin II, which was later shown to be identical to dormin. The name abscisic acid (ABA) was agreed by the two groups (Addicott *et al.*, 1968), yet it has become subsequently clear that ABA is not involved in abscission processes and its accumulation in abscising fruit may have been due to the stress associated with this process.

Several more groups of compounds with biological activity on plants were subsequently discovered, but there was initially some resistance to according them full plant hormone status, partly because it was difficult to assign them a physiological function. The brassinosteroids provide a prime example of this. The first member of this group to be chemically identified was brassinolide, which was isolated from oilseed rape (*Brassica napus*) pollen in the 1970s (Grove *et al.*, 1979). Brassinolide and other brassinosteroids were shown to have activity in bioassays, but it was not until a number of severely dwarfed GA-insensitive mutants were found to contain lesions in brassinosteroid biosynthesis or response (see Chapter 3) could the claim of brassinosteroids as endogenous growth regulators be no longer ignored. The



brassinosteroid signaling pathway is now better understood than for some of the longer established hormones and its receptor was one of the first to be identified.

Jasmonic acid and related compounds such as cucurbitic acid were identified as endogenous growth inhibitors in the 1970s and 1980s. Jasmonic acid has subsequently been shown to be involved in a number of physiological responses, particularly reproductive development and in resistance to insect herbivores and pathogens. There is also evidence that volatile metabolites of jasmonic acid, such as methyl jasmonate and *cis*-jasmane, may function in interplant or plant-insect communication (see Chapter 7). Pathogen resistance, particularly to biotrophic pathogens, is also mediated by salicyclic acid, a plant metabolite that is better known for its medicinal properties. Like jasmonic acid, salicyclic acid has been associated with a number of physiological processes in plants, the most spectacular of which is the induction of thermogenesis in the spadex of arum lily. However, its involvement in systemic acquired resistance has been the most extensively studied (see Chapter 8).

Lack of space prevents inclusion in this volume of a number of more recently discovered molecules, for which details of the signaling pathways are less advanced or for which hormonal activity is less clear. A signaling role for polyamines in plants was recognised recently but, although they participate in numerous developmental processes, their presence at relatively high concentrations suggests that they do not act strictly as hormones. The phyto-sulfokines, a group of sulphated pentapeptides, were isolated from rice cell suspension cultures and shown to stimulate cell division (Matsubayashi *et al.*, 1997), although their signal transduction pathway is unknown. Numerous other molecules from various sources have been shown to have often quite specific physiological effects. Recently the component of plant-derived smoke that stimulates seed germination was identified as a butenolide derivative with a striking structural similarity with strigol, a compound present in root exudates of many species that stimulates germination of the parasitic weeds *Striga* and *Orobanch*e (Flematti *et al.*, 2004). This raises the possibility that related compounds may have a hormonal function. The existence of a potentially new group of unidentified hormones that control branching has been indicated by genetic studies (reviewed in Schmitz & Theres, 2005). Components of the biosynthetic and signal transduction pathway for these hormones are encoded by the *MORE AXILLARY GROWTH* (*MAX*) genes of *Arabidopsis* and the *RAMOSUS* (*RMS*) genes of pea.

The absence of appropriate technology meant that progress in understanding the function of the hormones was initially slow and relied on observations of the effects of exogenous hormone applications. In some cases, the availability of hormone-deficient mutants was extremely helpful in determining physiological function and in investigating the hormone biosynthetic and catabolic pathways. The advent of molecular genetics and plant transformation, together with the development of sensitive and high throughput analytical techniques has enabled rapid advances in our understanding of hormone signaling such that most details of the metabolic pathways are known for all the major hormones and substantial progress has been made in elucidating their signal transduction pathways. Receptors have now been identified

for seven of the eight hormone classes covered in this volume. While the first three receptors to be discovered, those for ethylene, brassinosteroids and cytokinins are located on the plasma membrane, soluble receptors have been identified for auxin, GA and ABA. The recently discovered auxin and GA receptors participate fairly directly in the ubiquitin ligase-mediated degradation of transcriptional regulators, bypassing the need for a complex signaling cascade. Time will tell whether or not the membrane receptors predicted for these hormones by numerous earlier studies also exist.

Protein degradation is proving to be a common theme in plant hormone signal transduction, the hormone signal resulting in removal or stabilisation of transcriptional regulators, depending on the pathway. Another common feature is the presence of feedback regulation, in which transduction of the hormone signal results in modification, usually repression, of biosynthesis at the transcript level, providing a mechanism for hormone homeostasis. Moreover, it is also clear that there are complex interactions between the hormone pathways, acting on biosynthesis or signal transduction, that allow homeostasis at a higher level. Most developmental processes respond to several hormones, which mediate and integrate the different intrinsic and extrinsic cues that act on these processes. This volume includes chapters that consider the hormonal regulation of reproductive development (Chapter 10) and of seed development and germination (Chapter 11). A complete understanding of the hormonal control of development will need to take account of their interactions, the complexity of which may ultimately be fully understood only with the help of computer modelling.

This volume also includes a chapter on hormone distribution (Chapter 9), focusing on IAA, GAs and brassinosteroids, which differ considerably in their mobility. Among the hormones, only IAA is known to be actively transported, while other hormones may move by diffusion between cells or over longer distances in the vascular system. Some hormones undoubtedly act in some cases within the cell in which they are produced. Thus, as in animals, examples of autocrine, paracrine and even endocrine signaling can be found in plants. The debate about whether plant hormones can legitimately be called such on the basis of the original definition, which recognised only endocrine signals, has long since been abandoned as irrelevant. Plants have developed their own chemical signaling system, the importance of which for survival, development and response to the environment is becoming ever more apparent with increased understanding.

Peter Hedden and Stephen Thomas

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# 1 Absciscic acid synthesis, metabolism and signal transduction

Annie Marion-Poll and Jeffrey Leung

## 1.1 Introduction

Plants have evolved two notable innovations permitting their successful colonization from aquatic to terrestrial environments, where available water is often limited and sporadic. The first is the elaboration of the seed as a discrete developmental stage in which the life cycle can be temporarily halted in order to withstand desiccation. The second is the emergence of the stomatal complex and all of the attendant mechanisms in controlling CO<sub>2</sub> uptake and metabolite delivery driven by the transpiration stream. Absciscic acid (ABA) is widely known for its role in mediating seed maturation processes such as desiccation tolerance and dormancy. During vegetative growth, ABA is also the key hormone in regulating drought adaptive responses, especially stomatal closure. Studies of ABA-deficient mutants have contributed to the clarification of the biosynthesis pathway and to the analysis of the physiological roles of the hormone. Such mutants show reduced seed dormancy and are highly sensitive to dehydration. Reverse genetics and biochemical approaches have helped to identify the main enzymes of the catabolic pathways. With *Arabidopsis* as an easily tractable genetic and genomic model, precise descriptions of “how”, “when” and “where” ABA is synthesized and metabolized will soon be completely available.

Physiological evidence indicates that multiple sites of ABA perception exist and an ABA receptor has very recently been identified as the flowering regulator FCA, an RNA-binding protein (Razem *et al.*, 2006). Genetic and molecular approaches in *Arabidopsis* have contributed considerably in the characterization of signaling intermediates (Finkelstein & Rock, 2002; Finkelstein *et al.*, 2002; Nishimura *et al.*, 2004). A rough estimate suggests that around 100 mutants with altered ABA sensitivity are currently known, of which more than 20 have been isolated due to their altered germination or vegetative growth in the presence of exogenous ABA. The remaining mutants have come from other types of genetic screens based on misexpression of marker genes, reverse genetics, searches for modifiers of known ABA-sensitivity mutations or from unrelated work that ultimately revealed their altered sensitivity to several hormones including ABA. An irony of the success of this approach is that these signaling proteins are of such diverse presumptive functions as to render a unifying concept difficult (Finkelstein & Rock, 2002; Himmelbach *et al.*, 2003).

For many laboratories, the stomatal guard cell represents a propitious cellular system to explore the role of ABA and its interaction with other hormonal and

environmental signals (Schroeder *et al.*, 2001; Mäser *et al.*, 2003; Fan *et al.*, 2004; Roelfsema & Hedrich, 2005). Second messengers such as  $\text{Ca}^{2+}$ , pH, nitric oxide, cyclic ADP-ribose and certain phospholipids were shown to act as crucial relays. However, the improvement of biophysical techniques enabling guard cell membrane transport to be measured for the first time in intact plants has brought new interpretations challenging the significance of these second messengers (Roelfsema *et al.*, 2001; Levchenko *et al.*, 2005).

Despite the current lack of a unifying concept of ABA action, certain classes of mutants are nonetheless prevalent. Interestingly, some of their corresponding proteins seem to belong to the same complex suggesting foci or nexus of local signaling network. This review will concentrate on these classes of proteins for which understanding their functions in a physiological context is more complete. Complementary reviews of ABA synthesis and signaling outside the scope of this chapter are available (Finkelstein & Gibson, 2002; Finkelstein & Rock, 2002; Finkelstein *et al.*, 2002; Schwartz *et al.*, 2003a; Nambara & Marion-Poll, 2005; Taylor *et al.*, 2005).

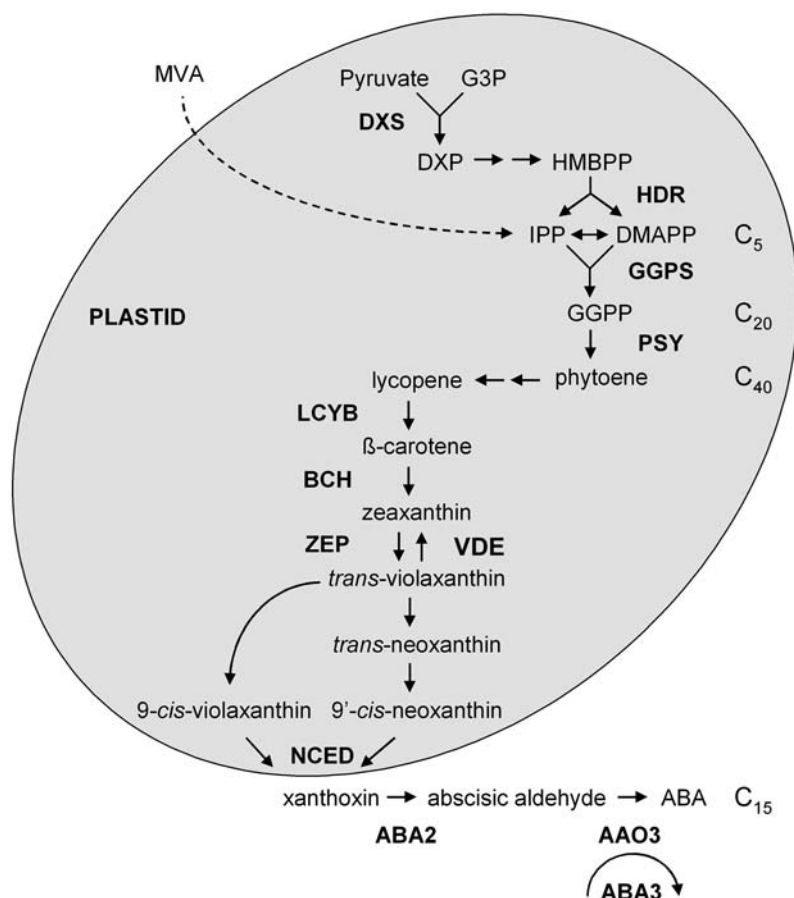
## 1.2 Biosynthesis and catabolism pathways

In the 1960s, one group studying leaf abscission in cotton-isolated abscisin, while another studying dormant buds in the sycamore-isolated dormin. These compounds turned out to be structurally identical and these two groups decided on the name “abscisic acid”. However, it has been argued that dormin may have been a more appropriate name after its wider-known physiological effects (<http://plantphys.info/Plant-Physiol/dormin.html>). In any case, abscisic acid or ABA has persisted.

### 1.2.1 Main early steps of ABA biosynthesis

ABA is formed by cleavage of carotenoids, which are derived from five-carbon precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Fig. 1.1). Plants synthesize IPP in the cytosol from acetyl-CoA via the mevalonic acid (MVA) pathway and in plastids from glyceraldehyde 3-phosphate and pyruvate via the methylerythritol phosphate (MEP) pathway (Rodríguez-Concepcion & Boronat, 2002). Since carotenoid synthesis takes place in plastids, the MEP pathway is the primary source of IPP precursor for ABA biosynthesis. An exchange of IPP and other downstream prenyl diphosphates can take place between cell compartments and MVA-derived cytosolic precursors might contribute to the production of carotenoids and ABA during specific developmental stages, such as in etiolated seedlings (Rodríguez-Concepcion *et al.*, 2004).

Phytoene is the common precursor for all plant carotenoids. This  $\text{C}_{40}$  backbone molecule is formed by phytoene synthase (PSY) after condensation of two molecules of geranylgeranyl diphosphate (GGPP), the  $\text{C}_{20}$  intermediate molecule synthesized from IPP and DMAPP. Desaturation and isomerization reactions are necessary to



**Figure 1.1** *ABA biosynthesis pathway*. IPP and its isomer DMAPP are produced in plastids from glyceraldehyde 3-phosphate and pyruvate via the MEP pathway. In the cytosol, IPP derives from acetyl-CoA via the MVA pathway; the dashed arrow indicates that IPP can be translocated from the cytosol to the plastid. The first step of the MEP pathway produces DXP by DXS and the last step, leading to IPP and DMAPP, is catalyzed by HDR. Then two successive reactions are necessary to form the first carotenoid, phytoene, catalyzed by GGPS and PSY. Desaturation and isomerization reactions (not shown) lead to lycopene, which is then converted to zeaxanthin, by LCYB (lycopene β-cyclase) and BCH (β-carotene hydroxylase). Its conversion into all-*trans*-violaxanthin is catalyzed by ZEP. Under high light a reverse reaction occurs in chloroplasts catalyzed by VDE (violaxanthin de-epoxidase). Genes encoding enzymes for the synthesis of *cis*-isomers of violaxanthin and neoxanthin have not been characterized. Carotenoid cleavage is catalyzed by a family of NCED to form xanthoxin, which is converted into ABA by ABA2 and AAO3. Sulfuration of AAO3 molybdenum cofactor by ABA3 is necessary for enzyme activity.

produce the all-*trans*-lycopene, through the formation of conjugated double bonds that constitute the chromophore in carotenoid pigments. β-carotene is formed by cyclization of lycopene, and its further hydroxylation, then leads to the formation of zeaxanthin, the first oxygenated carotenoid precursor of ABA.

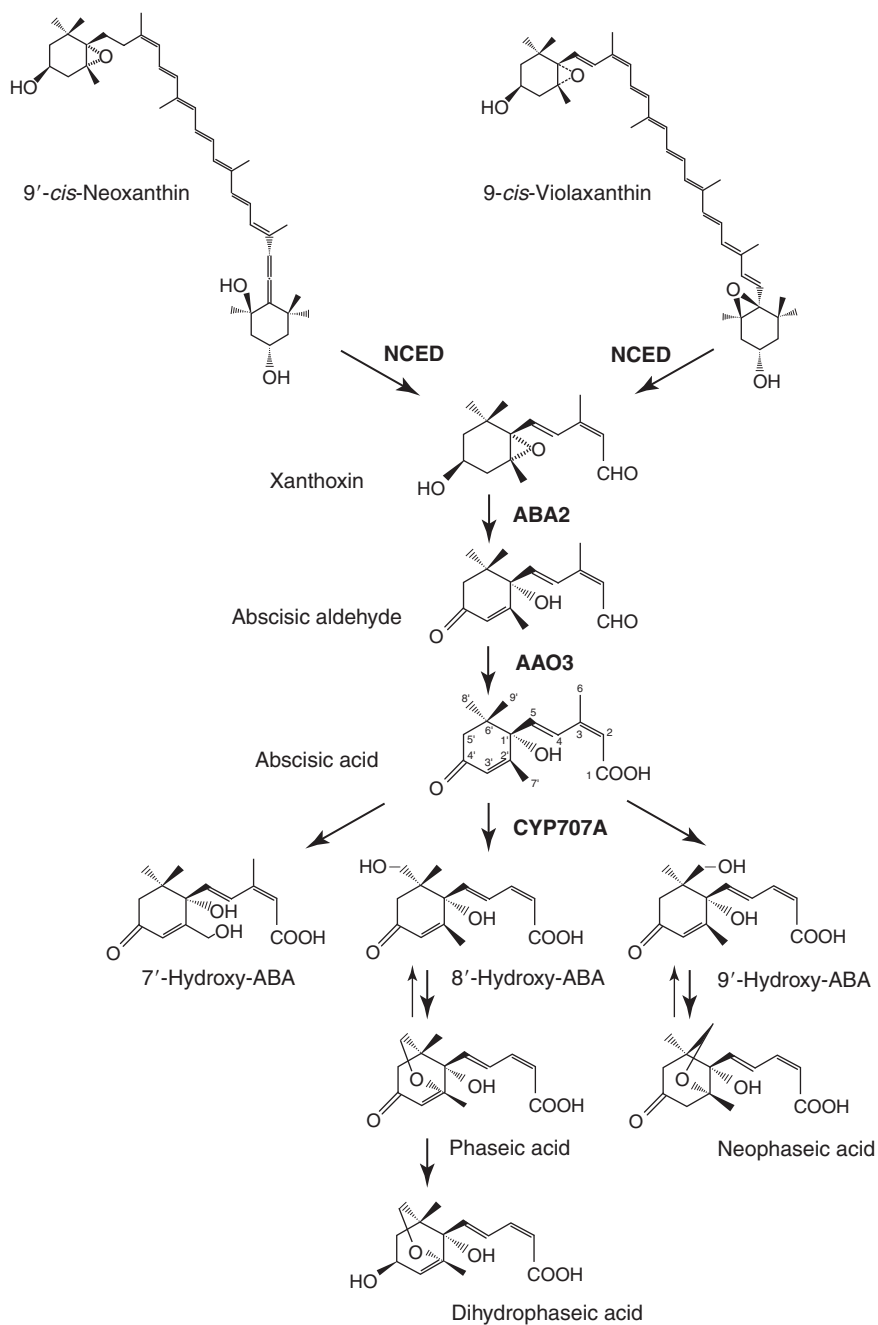
Zeaxanthin epoxidase (ZEP) converts *trans*-zeaxanthin into *trans*-violaxanthin by epoxidation of the two  $\beta$ -rings at each end of the molecule, via the formation of the mono-epoxidized intermediate antheraxanthin. *In vitro* activity of this enzyme requires ferredoxin as a reductant to catalyze the introduction of molecular oxygen into zeaxanthin (Bouvier *et al.*, 1996; Marin *et al.*, 1996). The enzymatic steps from *trans*-violaxanthin to the formation of *cis*-isomers of violaxanthin and neoxanthin have not yet been elucidated (Nambara & Marion-Poll, 2005). However the recent identification of mutants lacking neoxanthin isomers and the cloning of the corresponding gene will help to elucidate the last biosynthetic steps remaining to be characterized.

Genes encoding the biosynthetic enzymes described above have been cloned in various species (Eisenreich *et al.*, 2004; Fraser & Bramley, 2004). Carotenoids are highly abundant in chloroplasts, so that in green tissues ABA only represents a minor derivative of the carotenoid pathway. Therefore the involvement of the early biosynthetic genes in the regulation of ABA accumulation has not been thoroughly studied. Nevertheless, overexpression of genes encoding enzymes of the MEP and carotenoid pathway in non-chlorophyll-containing tissues, with low carotenoid levels, identified three that might limit ABA synthesis in *Arabidopsis*. Thus, overexpression of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), PSY and ZEP raised ABA levels in seeds, causing increased dormancy (Frey *et al.*, 1999; Estevez *et al.*, 2001; Lindgren *et al.*, 2003). More recently, enhanced dormancy was also reported in transgenic seeds overexpressing the gene encoding hydroxymethylbutenyl diphosphate reductase (HDR), the last enzyme of the MEP pathway that catalyzes the formation of both IPP and DMAPP (Botella-Pavia *et al.*, 2004). This suggests that HDR might also catalyze a limiting step for carotenoid and ABA synthesis.

### 1.2.2 Epoxy-carotenoid cleavage

The synthesis of xanthoxin results from the cleavage of 9'-*cis*-neoxanthin and 9-*cis*-violaxanthin, and is the last plastidial enzymatic step (Figs. 1.1 and 1.2). The cleavage of *cis*-xanthophylls by nine-*cis*-epoxycarotenoid dioxygenase (NCED) is the first committed step for ABA synthesis in the pathway. The maize VP14 recombinant protein encoded by the first cloned *NCED* gene was able to cleave 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin but not *trans*-xanthophyll isomers (Schwartz *et al.*, 1997; Tan *et al.*, 1997). The higher abundance of 9'-*cis*-neoxanthin compared to 9-*cis*-violaxanthin and the enzyme kinetics suggested that 9'-*cis*-neoxanthin might be the major substrate *in vivo* (Schwartz *et al.*, 2003b). Enzyme activity requires iron and oxygen to form a *cis*-isomer of xanthoxin. The *NCED* gene family in *Arabidopsis* is composed of nine members (Schwartz *et al.*, 2003a), of which five might be involved in ABA biosynthesis (Iuchi *et al.*, 2001; Tan *et al.*, 2003). As expected, mutants with reduced in or increased amounts of *AtNCED3*, *AtNCED6* and *AtNCED9* transcripts show corresponding alterations in cellular content of ABA (Iuchi *et al.*, 2001; Lefebvre *et al.*, 2006). The isoforms *AtNCED2*, *AtNCED3* and *AtNCED6*





**Figure 1.2** *Synthesis of ABA starting from carotenoid cleavage, and its catabolism by hydroxylation. The 8'-hydroxylation is thought to be the predominant pathway for ABA catabolism. Hydroxy groups of ABA and its catabolites are targets for conjugation.*

are localized in both thylakoid and stroma fractions, AtNCED5 is exclusively in the thylakoid fraction, while AtNCED9 is in the stroma (Tan *et al.*, 2003). As carotenoid substrates are located inside plastid membranes, the membrane-binding affinity might influence NCED activity through interaction with other membrane proteins or heterodimer formation among NCED isoforms. The four other enzymes, more distantly related to maize VP14, have been named AtCCD for carotenoid cleavage dioxygenase. Interestingly, AtCCD7 and AtCCD8 might be involved in the sequential cleavage of  $\beta$ -carotene in the synthesis of an unknown mobile signal that inhibits shoot branching (Schwartz *et al.*, 2004; Leyser, 2005).

### 1.2.3 The conversion of xanthoxin to ABA

ABA is produced in the biologically active *cis*-configuration from xanthoxin by two enzymatic steps (Figs. 1.1 and 1.2). The conversion of xanthoxin to abscisic aldehyde is catalyzed by an enzyme similar to short-chain dehydrogenases/reductases, named SDR1, and is encoded by the *AtABA2* gene in *Arabidopsis* (Rook *et al.*, 2001; Cheng *et al.*, 2002; Gonzalez-Guzman *et al.*, 2002). *In vitro* SDR requires NAD as a coenzyme for activity and transient expression of a GFP fusion protein in *Arabidopsis* protoplasts showed that it is cytosolic.

The oxidation of the ABA aldehyde to the carboxylic acid is the final step of ABA biosynthesis, catalyzed by abscisic aldehyde oxidase (AAO). In *Arabidopsis*, four homologous AAO genes have been cloned; but only one of them, *AAO3*, encodes a protein that has proven activity on abscisic aldehyde (Seo *et al.*, 2000a). Mutations in the *AAO3* gene result in an ABA-deficient mutant phenotype, confirming its biosynthetic function (Seo *et al.*, 2000b; Gonzalez-Guzman *et al.*, 2004). Since the seed phenotype of *aao3* is mild compared to other ABA-deficient mutants affected in unique genes, the involvement of other AAO isoforms in ABA synthesis has been investigated (Seo *et al.*, 2004). Mutations in *AAO1* or *AAO4* did not result in a detectable ABA-deficient phenotype, but combined with the *aao3* mutation, they enhanced ABA deficiency in the double mutant seeds. Therefore *AAO1* and *AAO4* proteins have been suggested to have a negligible role in ABA biosynthesis in wild-type seeds but contribute to some extent to ABA synthesis in the *aao3* mutant background. AAO proteins have a dimeric structure and contain iron–sulfur, FAD binding and molybdopterin domains. The activation of the molybdenum cofactor (Moco), necessary for AAO activity, requires the addition of a sulfur atom to the Mo center, which is catalyzed by a Moco sulfurase, named *ABA3* in *Arabidopsis* and *FLACCA* in tomato, both of which share homology with NifS proteins (Bittner *et al.*, 2001; Xiong *et al.*, 2001a; Sagi *et al.*, 2002).

### 1.2.4 ABA catabolism

In most tissues, the major catabolic route consists of the hydroxylation at the 8' position to form the unstable intermediate, 8'-hydroxy ABA, which is then cyclized to form phaseic acid (PA) (Fig. 1.2). Reduction of PA at the 4' position produces

mainly dihydrophaseic acid (DPA) and smaller amounts of *epi*-DPA. Two other minor oxidation pathways have been identified, yielding either 7'-OH ABA or 9'-OH ABA. The cyclized form of 9'-OH ABA has been found and named neoPA (Zhou *et al.*, 2004). ABA and its metabolites can be further conjugated, mainly to glucose, and ABA-glucose ester (ABA-GE) is the most widespread conjugate (Cutler & Krochko, 1999).

The catabolites 8'-OH ABA, PA, 7'-OH ABA, 9'-OH ABA and neoPA retain some hormonal activity; PA and neoPA being less active than hydroxy ABA forms (Zhou *et al.*, 2004). These various catabolites might therefore have biological roles. Complete inactivation occurs only after DPA formation. Conjugated forms of ABA have also been reported to be physiologically inactive, but might be involved in long-distance transport of the hormone; however, transport mechanisms and conjugate hydrolysis remain to be characterized (Sauter *et al.*, 2001).

ABA 8'-hydroxylase catalyzes the first step of the major catabolic route and has been shown to be a cytochrome P450 mono-oxygenase (Krochko *et al.*, 1998). In *Arabidopsis*, it is encoded by the four members of the *CYP707A* family (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Analysis of recombinant protein activity indicated that all four enzymes were able to convert ABA into 8'-OH ABA, but were not involved in the formation of other hydroxylated catabolites or in the cyclization of 8'-OH ABA to PA. The mild phenotypes of *cyp707a2* and *cyp707a3* mutants suggest functional redundancy, although increased ABA levels in *cyp707a2* mutant seeds confirm gene function (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Enzymatic steps downstream of the ABA oxidative pathway have not yet been identified.

The ABA glucosyl transferase converting ABA into ABA-GE belongs to a large group of glycosyltransferases. Proteins have been identified in adzuki bean and in *Arabidopsis* that were capable of *in vitro* glucosylation of ABA. The adzuki bean recombinant protein, AOG, has been shown to convert 2-*trans*-ABA, a biologically inactive isomer, more efficiently than the natural (+)-ABA (Xu *et al.*, 2002). Eight glucosyl transferases from *Arabidopsis* were found to produce ABA-GE from a racemic mixture of ABA enantiomers *in vitro* (Lim *et al.*, 2005). However only one of them, UGT71B6, was exclusively active on the natural enantiomer. In addition, this enzyme did not glucosylate ABA catabolites (Priest *et al.*, 2005). The *in planta* function of these putative ABA glucosyl transferases has still to be confirmed.

### 1.3 Regulation of ABA synthesis and metabolism

#### 1.3.1 Developmental regulation

##### 1.3.1.1 Vegetative tissues

ABA is often described as a growth inhibitor because reduced growth under stress conditions is correlated with increased ABA levels and exogenously applied ABA prevents germination and seedling growth. However, the stunted phenotype of ABA-deficient mutants in the absence of stress suggests a role for ABA as a growth

promoter (Sharp *et al.*, 2000; Cheng *et al.*, 2002). Additionally, ABA may also have a positive role in organogenesis. Reduced ABA levels in mutants or in transgenic plants results in developmental defects such as altered organization of the mesophyll and stomatal morphogenesis (Wigger *et al.*, 2002; Barrero *et al.*, 2005). Morphological alterations and growth reduction in ABA-deficient mutants have been partly attributed to increased ethylene levels. This is particularly clear in tomato mutants, which exhibit characteristic symptoms of ethylene excess, such as leaf epinasty and adventitious rooting, together with higher rate of ethylene evolution (Sharp *et al.*, 2000; Thompson *et al.*, 2004). Nevertheless, phenotypic analysis of double mutants exhibiting both ABA deficiency and ethylene insensitivity indicated that ABA maintained shoot development also by an ethylene-independent mechanism (LeNoble *et al.*, 2004). ABA is therefore both a growth promoter and an inhibitor depending on its level and site of accumulation.

ABA is mobile and it is detected throughout plant development in all tissues. In vegetative organs of well-watered plants, vascular bundles might be the predominant site of ABA synthesis, since concomitant expression of *AtNCED3*, *AtABA2* and *AAO3* genes has been detected (Cheng *et al.*, 2002; Tan *et al.*, 2003; Koiwai *et al.*, 2004). Furthermore, immunolocalization and *in vivo* imaging of ABA-active pools have confirmed the presence of ABA in these tissues (Wachter *et al.*, 2003; Christmann *et al.*, 2005). It has been suggested that the vascular tissue-specific gene expression facilitates transport of ABA.

In *Arabidopsis* roots, *AtNCED2* and *AtNCED3* expression was detected in the pericycle at the sites of lateral root initiation (Tan *et al.*, 2003). Moreover, high levels of *AAO3* expression was found in the main and lateral root tips and in vascular bundles, whereas *AtABA2* transcripts were detected in branching points of lateral and mature roots (Cheng *et al.*, 2002; Tan *et al.*, 2003; Koiwai *et al.*, 2004). These highly localized expression patterns of the biosynthesis genes indicate that ABA may stimulate or retard root development in response to endogenous or exogenous signals. In maize, for example, ABA accumulation has been reported to maintain primary root elongation under stress conditions whereas ABA would contribute to the inhibition of root branching by nitrate (Sharp *et al.*, 2000; Signora *et al.*, 2001; De Smet *et al.*, 2003).

### 1.3.1.2 Reproductive organs

Compared to seeds, the regulatory role of ABA in flower and gametophyte development is less documented. Transcripts of biosynthetic and catabolic genes have been detected in reproductive tissues. In particular, *AtABA2* and *AtNCED6* genes are highly expressed during later stages of pollen development, suggesting their possible role in pollen desiccation tolerance (Cheng *et al.*, 2002; Tan *et al.*, 2003). In addition, *AtNCEDs* and *AtABA2* expression has been detected in connective organs, such as anther filaments, flower pedicels and seed funicles. This suggests that ABA might be involved in reproductive organ abscission and/or in gametophyte and seed development.

During seed development, ABA is furnished largely by maternal tissues, and only a small fraction is synthesized in embryonic tissues during later stages

(Karssen *et al.*, 1983; Groot *et al.*, 1991). ABA levels are low during the early stages, as would be necessary to avoid seed abortion (Cheng *et al.*, 2002; Tan *et al.*, 2003). At the end of embryogenesis, ABA levels rise and block embryo growth, thus preventing vivipary. ABA levels are maximal during the maturation phase when the hormone is involved in the stimulation of reserve storage and the induction of desiccation tolerance. Analysis of ABA-deficient mutants produced from reciprocal crosses has also proven that embryonic ABA is specifically involved in the induction of seed dormancy (Karssen *et al.*, 1983; Groot *et al.*, 1991). In the final phase, seed water content and ABA levels decrease to low levels in mature dry seed.

As mentioned above, epoxycarotenoid synthesis probably contributes to the regulation of ABA synthesis in seeds as they are more dormant when the *ZEP* transcript is overexpressed (Frey *et al.*, 1999) and temporal regulation of *ZEP* transcript is closely correlated with ABA accumulation (Audran *et al.*, 1998). However, the ubiquitous expression of *ZEP* suggests that downstream steps such as carotenoid cleavage might control the tissue specificity of ABA synthesis in seeds (Audran *et al.*, 2001). Current evidence indicates that *AtNCED5*, *AtNCED6* and *AtNCED9* are highly and differentially expressed in embryonic tissues during *Arabidopsis* seed development (Tan *et al.*, 2003; Lefebvre *et al.*, 2006). In particular, *AtNCED6* expression is specific to the endosperm, whereas *AtNCED9* is expressed in both endosperm and embryo (Lefebvre *et al.*, 2006). Furthermore, analysis of mutant seed phenotypes indicates that ABA synthesized in both the embryo and the endosperm is responsible for seed dormancy induction.

Genes encoding downstream enzymes (*ABA2*, *AAO3* and *CYP707A*) are reported to be expressed in developing seeds, but no detailed tissue-specific analysis is yet available. Nevertheless, the catabolic *CYP707A* genes may contribute to decreasing ABA levels during the desiccation phase, since the ABA content in dry seeds of the *cyp707a2* mutant is higher than that in the wild-type seeds (Kushiro *et al.*, 2004). Seed germination is preceded by a decrease in ABA levels, with a concomitant accumulation of catabolite. In contrast, dormant seeds maintain high ABA levels during seed imbibition (Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004). *CYP707A2* might also be involved in the rapid decrease of ABA during early seed imbibition, since hydrated *cyp707a2* seeds maintain high ABA levels and exhibit increased dormancy compared to wild type. Therefore ABA hydroxylation is likely to be involved in the regulation of seed dormancy in *Arabidopsis*.

### 1.3.2 Regulation in response to abiotic stresses

Drought, salt and osmotic stresses have been shown to activate ABA synthesis, while its catabolism is induced to a lesser extent. In contrast, the latter has a major role in decreasing ABA levels upon stress release.

Differential regulation of *ZEP* expression upon drought stress has been observed in *Nicotiana plumbaginifolia* and tomato roots but not in leaves (Audran *et al.*, 1998; Thompson *et al.*, 2000). In *Arabidopsis*, drought, salt and osmotic stresses increase *ZEP* expression in both roots and shoots (Xiong *et al.*, 2002a), whereas in

other reports drought-induced upregulation has been observed in roots and not in leaves (Audran *et al.*, 2001; North *et al.*, 2005). In any event, the high carotenoid levels found in photosynthetic tissues are not likely to limit ABA synthesis, and transgenic plants overexpressing *ZEP* do not exhibit increased ABA levels nor improved water stress tolerance (Borel *et al.*, 2001).

The importance of the cleavage step in the regulation of ABA biosynthesis by drought stress is well documented (Xiong & Zhu, 2003; Schwartz *et al.*, 2003a; Nambara & Marion-Poll, 2005). The induction of *NCED* gene expression has been observed in various species, both in roots and in leaves. In addition, both transcript and protein levels increase prior to ABA accumulation (Qin & Zeevaart, 1999), whereas for *ZEP* and *AAO3*, only their transcript but not protein levels are affected (Seo *et al.*, 2000a; North *et al.*, 2005). Interestingly, in *Arabidopsis*, only *NCED3* gene is highly activated under drought stress (Iuchi *et al.*, 2001; Tan *et al.*, 2003). In accordance, the corresponding mutants are more sensitive to dehydration than wild type, whereas *Atnced6* and *Atnced9* mutants are unaffected (Iuchi *et al.*, 2001; Ruggiero *et al.*, 2004; Lefebvre *et al.*, 2006). In contrast to *ZEP*, *AtNCED3*, *AAO3* and *AtABA3*, *AtABA2* is apparently the only gene of the pathway that is not responsive to drought and salt stress (Cheng *et al.*, 2002; Gonzalez-Guzman *et al.*, 2002; Xiong *et al.*, 2002a). However results on *AtABA2* expression induced by mannitol are contradictory and further studies will be needed.

The increase in ABA levels in response to stress is achieved in part by its higher synthesis relative to its degradation rate. In *Arabidopsis*, the *CYP707A* transcript accumulates under drought, salt and osmotic stress (Kushiro *et al.*, 2004; Saito *et al.*, 2004) but the level is still lower than that for *NCED3*. Upon rehydration the relative levels of the two transcripts are reversed with a notable increase in PA.

The sites of ABA synthesis under water stress conditions are controversial. It is thought to be synthesized in the roots when soil dries and subsequently translocated to the shoot for regulation of transpiration (Wilkinson & Davies, 2002). However *in vivo* imaging of ABA pools indicated that synthesis in *Arabidopsis* shoots might precede that in roots (Christmann *et al.*, 2005). As previously mentioned, ABA synthesis genes are expressed in vascular tissues of turgid plants (Tan *et al.*, 2003; Koiwai *et al.*, 2004). This restricted pattern of expression might be maintained under stress conditions, since *AtABA2* expression was significantly increased by osmotic stress in these tissues, and high ABA levels were detected (Cheng *et al.*, 2002; Christmann *et al.*, 2005). Furthermore, *AtNCED2*, *AtNCED3* and *AAO3* expression has been observed in guard cells of well-watered plants and *AAO3* was activated under dehydration (Tan *et al.*, 2003; Koiwai *et al.*, 2004). ABA synthesis might occur in guard cells and be induced upon water stress (Koiwai *et al.*, 2004).

### 1.3.3 Regulation by endogenous signals and factors

Many biosynthetic pathways are regulated by end-product feedback, and this has been investigated for ABA. In the absence of stress, no variation of basal transcript levels of ABA biosynthesis genes has been observed in vegetative tissues or in dry

seeds of ABA-deficient mutants as compared to those in the wild type (Audran *et al.*, 2001; Cheng *et al.*, 2002; Xiong *et al.*, 2002a; Seo *et al.*, 2004). Nevertheless, upregulation of *AtNCED3* and *AtNCED5* was detected in developing siliques of *aa03* and *aba3* mutants, whereas transcript levels of other biosynthetic genes were similar when compared to the wild type (Seo *et al.*, 2004). Negative-feedback regulation might therefore occur, but this would be restricted to certain developmental stages and only affect the carotenoid cleavage step. Other lines of evidence suggest the existence of positive feedback. For example, application of exogenous ABA to *Arabidopsis* plants stimulates the expression of *AtZEP*, *AAO3* and *AtABA3*, but not that of *AtABA2* (Xiong *et al.*, 2001a; Cheng *et al.*, 2002; Xiong *et al.*, 2002a). Increased *AtNCED3* expression by ABA was also observed in certain accessions of *Arabidopsis*, whereas no upregulation was detected for the tomato *LeNCED1* or the cowpea *VuNCED1* (Iuchi *et al.*, 2000; Thompson *et al.*, 2000). These contrasting results suggest that positive regulation by ABA is not a general and evolutionary conserved mechanism. However it might be important for stress adaptation as suggested by Xiong and Zhu (2003) since an initial induction of ABA synthesis under stress would further stimulate ABA accumulation through a positive-feedback loop. Regulation of the catabolic pathway appears to be less controversial. First, biochemical evidence indicated that ABA 8'-hydroxylase activity was enhanced by ABA in cultured cells of several species (Windsor & Zeevaart, 1997; Cutler & Krochko, 1999). Second, accumulation of the *CYP707A* transcript has also been shown to be induced by ABA (Saito *et al.*, 2004). Third, increased ABA accumulation in transgenic plants overexpressing *NCED* transcripts was correlated with higher PA levels (Qin & Zeevaart, 2002). Therefore, it appears that ABA largely controls its own endogenous levels through a positive-feedforward regulation of catabolic enzymes.

The promoters of several ABA biosynthesis genes contain putative ABA- and drought-responsive elements (Xiong & Zhu, 2003; Thompson *et al.*, 2004). Many signaling components have been identified that function in ABA-dependent or -independent pathways in stress response (see below). Among these, calcium may act as second messenger and recent evidence suggested that a putative calcium sensor, Calcineurin B-Like 9 (CBL9), might be a negative regulator of the positive-feedback loop by which ABA regulates its own synthesis (Pandey *et al.*, 2004). Like *cbl9*, the *sad1* mutant is hypersensitive to ABA and osmotic stress. In contrast to *cbl9* or wild type, increases in ABA levels are more modest in the *sad1* mutant under stress (Xiong *et al.*, 2001b). The *SAD1* locus encodes a polypeptide similar to multifunctional Sm-like snRNP proteins required for mRNA splicing, export and degradation. As compared to the wild type, the *AAO3* and *AtABA3* transcripts are reduced in the *sad1* mutant. These results suggest that *SAD1* might be involved in the transcript turnover of an ABA biosynthesis regulator that would control ABA homeostasis through the positive-feedback loop.

Besides ABA, glucose is also a potential regulator of ABA biosynthesis because certain ABA-deficient (and sensitivity) mutants are also altered in glucose sensitivity (Leon & Sheen, 2003). Most ABA biosynthesis genes were found to be upregulated



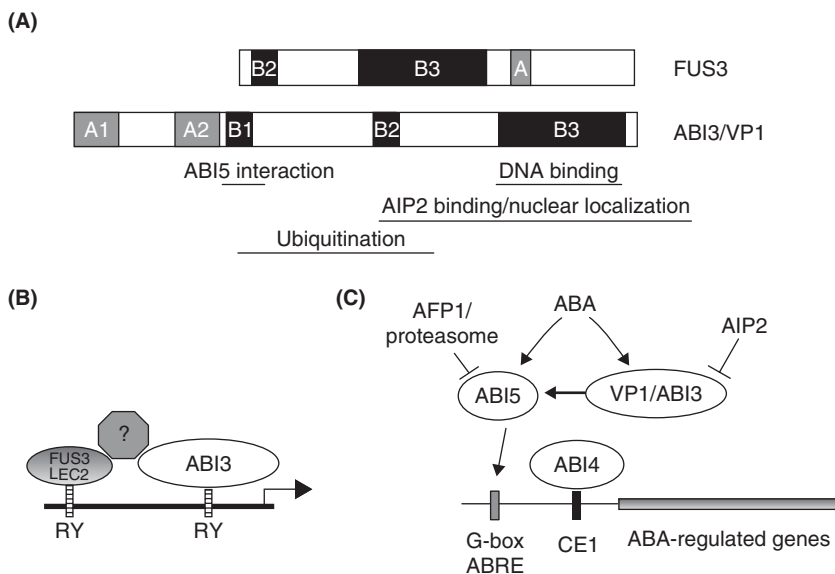
by glucose and this induction was reduced in an ABA-deficient background, indicating that glucose and ABA may act synergistically (Cheng *et al.*, 2002). A putative glutamate receptor, AtGLR1.1, has been recently proposed to function as a signaling component for the regulation of ABA synthesis in response to sugars (Kang & Turano, 2003; Kang *et al.*, 2004).

ABA has been shown to interact with several hormones to regulate various aspects of plant development and adaptive responses to biotic and abiotic stresses. However no strong evidence is yet available showing a direct effect of other hormones on ABA synthesis or catabolism. Several *cis*-acting regulatory elements have been found in the *LeNCED1* promoter, but their proposed function in ethylene, GA or auxin response still remains to be proven (Thompson *et al.*, 2004). In addition, several reports have indicated that alterations in ethylene production or signaling affected ABA accumulation, but only one study reported a slight overexpression of the *ZEP* transcript in the ethylene-insensitive mutant *ein2* correlating to increased ABA levels (Ghassemian *et al.*, 2000). Effect of gibberellins (GA) on ABA levels are even less documented; nevertheless, high ABA levels have been detected in the GA-constitutive response mutant *slender* of rice (Ikeda *et al.*, 2002) and GA, together with brassinosteroids, has been shown to regulate ABA catabolic gene expression (Saito *et al.*, 2004). Recently, a common regulator of ABA and GA synthesis has been identified (Gazzarrini *et al.*, 2004). As detailed below, the transcription factor *FUS3* has been shown to regulate seed development by positively regulating ABA accumulation and downregulating GA synthesis.

#### **1.4 ABA signaling in seed maturation processes: proteolysis and combinatorial protein interactions**

During seed maturation, storage proteins accumulate to high levels and their regulation has served as a model in understanding the role of gene transcription in seed maturation. The maize transcription factor, VP1, was identified by transposon-tagging screens of viviparous embryos. VP1 was shown to bind *in vitro* to the core sequence CATGCA that constitutes part of the so-called RY elements found in the promoters of presumptive target genes (Suzuki *et al.*, 1997). The DNA binding requires an intact B3 domain in the protein that is also conserved in over 40 other proteins in *Arabidopsis* (Fig. 1.3a). The *Arabidopsis* orthologue of VP1 is ABSCISIC-ACID-INSENSITIVE 3 (ABI3), and its B3 domain has also been shown to be required for gene activation through the RY element using a reporter promoter *napA* of *Brassica napus* expressed in transgenic tobacco (Ezcurra *et al.*, 2000). More recently, direct *in vitro* binding of this B3 domain to the RY elements has been demonstrated (Mönke *et al.*, 2004). Comparing the protein sequences of VP1 and ABI3 has revealed that, besides B3, they also share several other conserved domains (Fig. 1.3a). The domain B1 in ABI3 was shown to interact directly with ABI5 in the yeast two-hybrid test (Nakamura *et al.*, 2001). Interaction between these two homologous proteins in rice was also reported previously (Hobo *et al.*, 1999). *ABI5* encodes a bZIP-type





**Figure 1.3 Major seed transcription regulators.** (a) Modular structures of FUS3 and VP1/ABI3 proteins with conserved domains highlighted. Assigned functions of different parts of the proteins are noted below. (b) ABI3, FUS3 and LEC2 can bind to the RY element in target promoters. ABI3 can synergistically interact with FUS3 (Parcy *et al.*, 1997) to control seed development but there is no yet molecular evidence for cooperative binding of the two transcription factors to target promoters. (c) Gene transcription mediated by ABI3 and ABI5. ABI3 can function as a co-activator and may co-opt ABI5 which can then bind to the G-box or the similar ABRE motif in target promoters. There are 43 B3-domain and 81 bZIP-type transcription factors in the *Arabidopsis* genome, but most likely only a small fraction of each class is involved in ABA signal relay. ABI3 and ABI5 are regulated, at least in part, by ubiquitin-mediated proteolysis (modified from Suzuki *et al.*, 2003).

transcription factor, mutations of which were detected in genetic screens based on ABA-resistant germination and no expression of an *Em* promoter-*uidA* gene reporter (Finkelstein & Lynch, 2000; Carles *et al.*, 2002). Both *abi3* and *abi5* seeds also display mild glucose resistance suggesting that they may participate in sugar signaling, even though they had never been identified as such in mutant screens (Finkelstein & Gibson, 2002). Furthermore, mutant analysis and cross-complementation studies using transgenic plants defined ABI5 acting downstream of ABI3 in the same seed developmental pathway (Lopez-Molina *et al.*, 2002).

Both ABI3 and ABI5 are unstable proteins. ABI3 is specifically targeted *in vivo* by the E3 ligase AIP2 (Zhang *et al.*, 2005). *In vitro* binding assays showed that AIP2 has significant affinity for the B2 and B3 domains of ABI3, whereas the ubiquitination sites are localized to the region subsuming the B1 and B2 domains. Thus, the substrate domain imparting interaction specificity with its E3 ligase in this case is distinct from that of ubiquitination. Proteolysis of ABI5 is similarly thought to release the seedling from post-germination arrest caused by adverse environmental

conditions (Lopez-Molina *et al.*, 2001). ABI5 is targeted by the ABI Five binding Protein (AFP) to proteasomes that also include CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) (Lopez-Molina *et al.*, 2003). In addition to AFP, the stability of ABI5 seems to be also regulated by the proteasome itself. REGULATORY PARTICLE NONATPASE 10 (RPN10) is a subunit of the 19S regulatory complex of the 26S proteasome (Smalle *et al.*, 2003) and the corresponding mutant accumulates ABI5 (Hare *et al.*, 2003; Smalle *et al.*, 2003). Conversely, ABI5 is stabilized by phosphorylation by an ABA-activated 42-kDa kinase that could be MAP kinase 3 (MPK3) (Lu *et al.*, 2002).

The maize *VP1* gene can complement the *Arabidopsis* mutant (Suzuki *et al.*, 2001). Therefore, ectopic expression of *VP1* in *Arabidopsis* has been used as a proxy for *ABI3* in order to identify downstream genes (Suzuki *et al.*, 2003). There are 81 annotated genes encoding bZIP transcription factors in *Arabidopsis*, of which 37 were represented on the gene chips used in these experiments. The results showed that all 13 bZIP proteins in the same clade as ABI5 were activated by VP1 in an ABA-dependent manner. Analysis of promoters of these target genes revealed a subset of ABRE that are enriched in the 600-bp 5' upstream region. VP1 does not bind these elements directly, and may require recruitment of their own target bZIP proteins such as ABI5 for binding (Nakamura *et al.*, 2001) (Fig. 1.3b). These ectopic expression studies also revealed that VP1 is implicated in the potential suppression of two key negative regulators of ABA, *ABI1* and *ABI2*, which encode homologous protein phosphatases 2C (PP2Cs). In these studies, the PP2Cs belonged to the second largest class of genes that are ABA activated and yet VP1 suppressed. Thus, although VP1 has been extensively studied as transcriptional activator, it could also function as a repressor depending on the promoter context.

The *Arabidopsis* activators FUSCA 3 (FUS3) and LEAFY COTYLEDON 2 (LEC2) were identified as transcription factors that can activate the seed-specific albumin 2S promoter *At2S3* in a one-hybrid screen (Kroj *et al.*, 2003). Like *ABI3*, these are also B3-domain-type transcription regulators, and mutations in *FUS3* and *LEC2* cause precocious germination despite near-normal ABA sensitivity. Both proteins can activate a yeast reporter gene under the control of the RY-G box (CACGTGGC, which is similar to the ABRE) derived from *At2S3*, and *in vitro*, FUS3 can bind to the RY-G motifs involving at least its B3 domain (Mönke *et al.*, 2004). The expression of *At2S3* is strongly and consistently suppressed by mutations affecting FUS3, LEC2 and *ABI3*, suggesting that these proteins are likely *in planta* regulators. LEC2 and FUS3 regulate the GA biosynthetic gene *AtGA3ox2* by binding to the two RY motif in the promoter (Curaba *et al.*, 2004). However, there is no evidence indicating that these proteins directly interact in a complex (Mönke *et al.*, 2004), but the possibility that they may be tethered by other chromatin components cannot be excluded (Fig. 1.3c).

Mutations at *FUS3* cause premature germination as described above, but in seedlings they also lead to the precocious expression of post-embryonic traits such as trichomes on cotyledons and unscheduled expression of leaf-specific genes. It had been shown previously that the heterochronic phenotype of *fus3* can be suppressed

by exogenous ABA and compounds that inhibit GA biosynthesis (Keith *et al.*, 1994). By contrast, ectopic expression of *FUS3* under the control of the *MERISTEM LAYER 1* (*AtML1*) promoter was shown to incite abnormalities including glabrous lateral organs that resemble cotyledons (Gazzarrini *et al.*, 2004). The ectopic cotyledon phenotype is reversible by exogenous GA or mutations that block ABA biosynthesis, indicating that *FUS3* exerts its effects through actions of these hormones. ABA increases the stability of *FUS3*, while GA has the opposite effect (Gazzarrini *et al.*, 2004). Thus *FUS3* may regulate a feedback loop involving the action of each of these two hormones, which in turn influence the stability of *FUS3*.

## 1.5 Stress responses in vegetative tissues: the five major nexuses

### 1.5.1 ABA recognition sites and the search for the receptors

Genetic screens for mutants altered in hormone sensitivity have identified physiological receptors for brassinosteroids (Wang, Z.-Y. *et al.*, 2001), cytokinins (Heyl & Schmülling, 2003), ethylene (Bleecker, 1999), and recently auxin (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005) and gibberellins (Ueguchi-Tanaka *et al.*, 2005), but similar screens have not directly yielded a bonafide ABA receptor. Multiple ABA reception sites exist (see below), but the functional redundancy cannot be the sole explanation for this failure because many of the other receptors identified genetically also have closely related homologs. It is possible that ABA receptors have other cellular functions such that their role in ABA signaling may be too subtle to incite suspicion. An instructive example is the F-box-containing protein, TIR1, which directs target specificity in proteolysis mediated by the SCF complex. TIR1 turned out also to bind auxin directly (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005). Indeed, a protein that has been known prominently for its role in the control of flowering time has now been shown to be one of the elusive ABA receptors (see below).

Stomatal closure and the inhibition of stomatal opening are the most robust physiological responses to ABA. It also induces characteristic ion fluxes such as increase in  $[Ca^{2+}]_{\text{cyt}}$ , and ion channel activities. These easily quantifiable effects have made the guard cell the preferred model for searching ABA recognition sites. Beginning in the mid-1990s up until now, many experiments have been carried out including microinjecting ABA to the cytosol or applying it externally in different pH to vary its permeability across the plasma membrane. The balance of the results showed perception sites inside the cell as well as on the outside surface of the plasma membrane (MacRobbie, 1995; Levchenko *et al.*, 2005). ABA-binding sites on the plasmalemma of *Vicia* guard cells have been visualized by using biotinylated and biologically active ABA (Yamazaki *et al.*, 2003). In aleurone protoplasts, or *Arabidopsis* cultured cells, characteristic responses triggered by ABA applied as an impermeable conjugate also argued for a recognition site on the outside of the cells (Gilroy & Jones, 1994; Jeannette *et al.*, 1999).

Several cloned genes encoding ABA-binding proteins were published in the late 1990s (Finkelstein & Rock, 2002; Finkelstein *et al.*, 2002). However, there was no further evidence indicating that, *in planta*, these proteins functioned as saturable ABA-binding sites. A recent hunt for the ABA receptor has focused on a particular leucine-rich repeat receptor-like protein kinases (RPK) whose transcript can be stimulated transiently by ABA (Osakabe *et al.*, 2005). Several knockdown or knockout *rpkl* mutants showed reduced ABA sensitivity in seed germination, growth, stomatal closure as well as downregulation of many ABA-responsive genes. It is not yet known whether RPK1 binds directly to ABA or if it acts by associating with an ABA-binding receptor complex.

A 42-kDa protein has been affinity purified from the abaxial epidermis of *Vicia* leaves (Zhang *et al.*, 2002). Scatchard plot analysis of this protein showed an equilibrium dissociation constant of 21 nM which is close to the 19 nM calculated for the binding sites visualized on the cell surface (Yamazaki *et al.*, 2003). This ABA-binding protein showed stereospecificity in that (–) ABA and *trans*-ABA were incapable of displacing  $^3\text{H}$ -( $\pm$ ) ABA bound to the protein and ( $\pm$ ) ABA was less effective than (+) ABA in the competition. The binding of ABA to this protein seems physiologically relevant because pretreating the *Vicia* guard cell protoplasts with a monoclonal antibody raised against the 42-kDa protein significantly decreased the ABA-induced phospholipase D activity in a dose-dependent manner. It is known that blocking phospholipase D partially suppresses ABA-induced senescence as well as other ABA-, wounding- and osmotic stress-related responses (Wang, 2002).

A second ABA-binding protein from ABA-treated barley aleurone was isolated by expression cloning of a cDNA using an anti-idiotypic antibody (Razem *et al.*, 2004). This 52-kDa protein, ABAP1 was experimentally shown to be membrane associated. It is characterized by a WW domain (known to bind proline), which is found in over 100 animal and plant proteins, including FCA implicated in the control of flowering time in *Arabidopsis* (Razem *et al.*, 2004). Recombinant ABAP1 binds slightly less than one (+)-ABA per molecule at neutral pH. In aleurone membranes, it is very abundant being about 20% of the total proteins. ABAP1 is not expressed in leaves indicating that this putative receptor is limited to certain tissues or developmental stages.

A search in gene banks consistently turned up FCA from different plant species as the closest homolog of ABAP1 (AF127388). Although sequence conservation between these homologs is fairly modest (<40% overall) and FCA is a nuclear protein that binds RNA as part of its well-established function in controlling flowering time, Razem *et al.* (2006) remained undaunted in their conviction that FCA could be an ABA receptor. Their insightful deduction was indeed correct. ABA binds directly to an as-yet undefined motif near the C-terminus of FCA impeding its association through its WW domain with FY (a 3'-RNA processing factor). The mutant *fca* shows no alteration in any of the "classical" ABA responses such as stomatal closure and seed germination. Instead, this elegant work has uncovered the participation of ABA in the so-called autonomous flowering pathway.

### 1.5.2 Transcriptional network as the readout

Genomic tools have afforded global surveys of the genes as “readout” in response to the ABA signal. Analyses using microarrays containing 7000 cDNA suggest 3–4% of the genes in *Arabidopsis* may respond to ABA (Takahashi *et al.*, 2004). Regardless of the particular experiments or technique, roughly the same proportion of them are up- and downregulated by the hormone (Hoth *et al.*, 2003; Suzuki *et al.*, 2003). In general, over 50% of the genes fall into the “unknown” class indicating the rather sizable gap in our present knowledge concerning the functions of these responsive genes. About 20% of the known responsive genes code for products that would be involved in “cellular metabolism” and “homeostasis”, but some enrichment of “transcription factors” (Takahashi *et al.*, 2004) and “proteolysis” (Hoth *et al.*, 2003) are noticeable. Those frequently encountered in the “cellular metabolism or homeostasis” category include the late embryogenesis abundant (LEA) proteins, chaperones, enzymes for osmolyte production and detoxification. Adverse conditions also induce the expression of non-coding RNA, including many small interfering or micro RNAs that can form transient double-stranded regions with the target transcript (Sunkar & Zhu, 2004). These small RNAs are also expressed in “non-stress” growth conditions suggesting that they may also have other physiological roles. One major conclusion from the coding-RNA profiling studies is that genes responsive to ABA have a high probability of containing the ABRE motif (PyACGTGGC) in their promoter regions (Suzuki *et al.*, 2003; Yamaguchi-Shinozaki & Shinozaki, 2005), which are the major target of bZIP class of transcription factors (Choi *et al.*, 2000; Uno *et al.*, 2000) (Fig. 1.3b). Moreover, the ABRE motif is either paired, or associated with the CE1 or CE3 motifs (Coupling Elements) (Shen *et al.*, 1996). The CE1 motif, at least in maize, has been shown to be a target of the ERF/AP2-type transcription factor ABI4 (Fig. 1.3b) (Niu & Bate, 2002). *ZmABI4* is probably the ortholog of *ABI4* in *Arabidopsis*, as the former complements the *abi4* mutant.

A second ABA-dependent transcription pathway was revealed from the analysis of *rd22*, a drought-responsive gene that is a downstream target of the OST1 kinase-mediated pathway (see later) (Yoshida *et al.*, 2002). Important motifs for its proper regulation by ABA and drought are recognized by the MYC helix–loop–helix (CACATG) and MYB (TGGTTAG) classes of transcription factors. Furthermore, ABA-inducible MYC and MYB transcription factors may cooperate in the ABA-dependent expression of *rd22* (Abe *et al.*, 2003; Yamaguchi-Shinozaki & Shinozaki, 2005).

### 1.5.3 RNA metabolism

Genetic screens based on seed germination (Lu & Fedoroff, 2000; Hugouvieux *et al.*, 2001) or the use of the stress-activated promoter derived from *rd29A* as a reporter (Ishitani *et al.*, 1997) have produced a wealth of mutants affected in ABA responses. An interesting generality is that many of them suggest or have defects in RNA

**Table 1.1** Mutations affecting ABA signaling that implicate putative RNA-binding proteins

Mutations	Mutant phenotypes	Putative gene products	References
<i>fry 2</i>	Slight resistance to ABA and salt, increase sensitivity to cold	Product with similarity to RNA polymerase II C-terminal domain phosphatase and to DSRM (the prototype double-stranded RNA-binding motifs)	Xiong <i>et al.</i> (2002b)
<i>los4</i>	Cold acclimatization defect slight hypersensitivity of ABA	DEAD-box RNA helicase, regulating cold acclimation and cold signaling through at least CBF3-mediated regulons	Gong <i>et al.</i> (2002)
<i>sad1</i>	Defective in the positive feedback in drought-induced ABA biosynthesis	Product similar to the multifunctional Sm-like U6 small nuclear ribonucleoprotein in animals and yeast	Xiong <i>et al.</i> (2001b)
<i>abh1</i>	ABA hypersensitive	Nuclear RNA cap-binding protein	Hugouvieux <i>et al.</i> (2001)
<i>cbp80</i>	ABA insensitive	CBP80, RNA cap-binding protein	Mentioned in Xiong <i>et al.</i> (2001b)
<i>cbp20</i>	ABA hypersensitive in germination, reduced stomatal conductance, drought-tolerant	CBP20, RNA cap-binding protein	Papp <i>et al.</i> (2004)
<i>hyl1</i>	ABA hypersensitive	Double-stranded RNA-binding protein	Lu & Fedoroff (2000)

processing, including that of double-stranded RNA, coinciding with the observations of micro or small RNAs that are inducible by stress (above). The role of RNA metabolism in stress responses and in plant development has been the subject of several recent reviews and will only be summarized here (Table 1.1) (Fedoroff, 2002; Khun & Schroeder, 2003; Cheng & Chen, 2004; Riera *et al.*, 2005). Noteworthy is *fry2*, which causes a slight resistance to ABA and salt, but increased sensitivity to cold, and is mutated in a gene encoding a novel protein with domains similar to RNA polymerase II C-terminal domain phosphatase and to the prototype double-stranded RNA-binding motifs (DSRM) (Xiong *et al.*, 2002b). Another double-stranded RNA-binding protein, HYL1, which affects small RNA metabolism, has been implicated in some aspects of ABA signaling as well as other pleiotropic phenotypes (Lu & Fedoroff, 2000; Han *et al.*, 2004; Vazquez *et al.*, 2004). It is also intriguing that many of the predicted proteins show no common features that would suggest a molecular basis for their involvement in ABA signaling. For example, disruptions of genes encoding three mRNA cap-binding proteins ABH1 (Hugouvieux *et al.*, 2001), CBP80 (discussed in Xiong *et al.*, 2001b) and CBP20 (Papp *et al.*, 2004) led to altered sensitivity to ABA and stress. It is conceivable that some of these RNA-binding proteins regulate the decay rate of mRNAs encoding components that are involved in early or critical steps of the signaling chain. For example,

removal of the long 5'-untranslated leader of *ABI3* can dramatically improve expression of the reporter *ABI3-GUS* expressed under the *ABI3* promoter (Ng *et al.*, 2004).

### 1.5.4 Protein phosphatases 2C

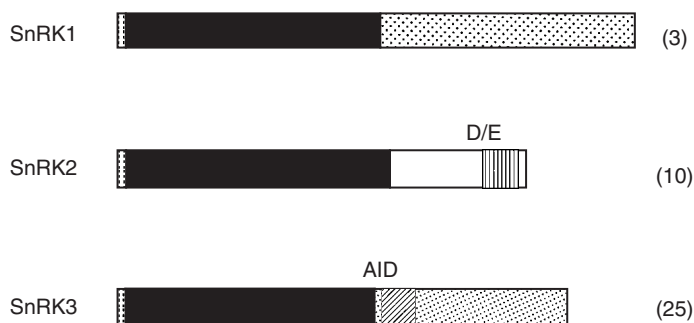
The other commonly encountered regulators are members of the PP2C family. Among the 69 annotated genes in the *Arabidopsis* genome, five are upregulated by ABA (Suzuki *et al.*, 2003). Experimental evidence indicates that ABI1, ABI2, AtPP2CA and AtPP2C-HA are negative regulators of the ABA signal. *ABI1* and *ABI2* have been by far the most studied genes; they act in a partially redundant manner in the resetting of a wide spectrum of ABA responses, including seed dormancy and germination, root growth and stomatal response (Gosti *et al.*, 1999; Merlot *et al.*, 2001). One possible explanation for the pleiotropic phenotypes is that these PP2Cs may complex with a wide variety of important signaling regulators. The homeodomain protein AtHB6 was the first identified target of ABI1 (Himmelbach *et al.*, 2002). Subsequently, ABI1 was also shown to bind weakly with the Sucrose non-fermenting Related Kinase, SnRK3.1, that participates in global stress responses (Guo *et al.*, 2002). However, it is not known whether these interacting proteins are direct substrates, since their dephosphorylation by ABI1 has not been demonstrated. ABI2 – less so with ABI1 – can interact with SnRK3.11, mutation of which renders the plant overly sensitive to salt stress (Guo *et al.*, 2002; Ohta *et al.*, 2003). As will be described in the section below on SnRKs, all of these components may be part of a larger complex in the global regulation of stresses.

A very exciting finding by Wang and colleagues that helps explain the pleiotropic phenotypes was that ABI1 can directly bind phosphatidic acid (Zhang, W. *et al.*, 2004), a lipid second messenger that transiently accumulates within minutes of applying a wide array of stress conditions (Testerink *et al.*, 2004). Phosphatidic acid mimics ABA action in aleurone, *Arabidopsis* cells and guard cells by inhibiting channel activity and inducing stomatal closure (Ritchie & Gilroy, 1988; Jacob *et al.*, 1999; Hallouin *et al.*, 2002), while an *Arabidopsis* mutant disrupted in the corresponding gene *PLD $\alpha$ 1* gene shows ABA-insensitive stomata (Zhang, W. *et al.*, 2004). The binding of phosphatidic acid to the N-terminal non-catalytic domain of ABI1 partially inhibits its *in vitro* activity. Since most of the phosphatidic acid in cells is confined to the plasma membrane, these authors suggested that the lipid may tether the PP2C by which its catalytic activity would be held in check. Indeed, addition of phosphatidic acid close to “physiological” level to *Arabidopsis* protoplasts leads to partial relocalization of the transiently expressed ABI1 to membranes (Zhang, W. *et al.*, 2004).

### 1.5.5 Sucrose non-fermenting-related kinases

Members of the conserved Sucrose Non-Fermenting (SNF) 1 serine/threonine protein kinase regulate glucose and stress signaling in eukaryotes. In yeast grown under glucose limitation, *SNF1* is required to de-repress all of the glucose-repressed





**Figure 1.4** The three classes of the SnRK in *Arabidopsis*. The N-terminal part of the protein is catalytic (black box), while the C-terminal extensions are likely to be regulatory being more divergent in sequences. The number of members in each family is shown in brackets (modified from Hrabak *et al.*, 2003). The D/E indicates an acidic patch and AID is the autoinhibitor domain.

genes. In animals, the homologous AMP-activated kinases function as key sensors of the balance between AMP and ATP, which indicates the metabolic state of the cell during stress (Hardie, 2004).

Plants contain a large number of kinases related to the prototype SNF1 from yeast (Hrabak *et al.*, 2003). Halford and Hardie (1998) have attributed to them the name SNF1-related kinases and in *Arabidopsis* these kinases have been divided into three subfamilies (SnRK1, SnRK2, SnRK3) based on distinctive structural features and, when applicable, potential regulatory functions attributed to their C-terminal domains (Hrabak *et al.*, 2003) (Fig. 1.4). There is evidence indicating that specific members from each of the three subfamilies are involved in mediating ABA signal transduction as part of their diverse repertoire of physiological functions.

The strongest evidence for ABA signaling is available for members of the SnRK2 family in several plant species. Nine of the ten in the *Arabidopsis* genome (Hrabak *et al.*, 2003) when expressed in transfected cells are inducible by hyperosmotic stress within minutes (Boudsocq *et al.*, 2004). The highly conserved N-terminal parts of these proteins are catalytic (Fig. 1.4), while the C-terminal domains are regulatory and divergent in sequence, except SnRK2.2, SnRK2.3, SnRK2.6, in which conserved blocks of amino acids are shared among them and with the ABA-activated protein kinase (AAPK) from *Vicia* (Mustilli *et al.*, 2002) (see below). These three SnRKs are responsive to exogenous ABA, in addition to mannitol and salt (Boudsocq *et al.*, 2004). The kinase SnRK2.6 has been identified genetically as the *open stomatal* (*ost1*) (Mustilli *et al.*, 2002) and *srk2e* (Yoshida *et al.*, 2002) mutations that block stomatal closure in response to ABA or to low humidity. The mutant stomata still responded normally to light and CO<sub>2</sub>, which stimulate opening and closing, respectively (Mustilli *et al.*, 2002), suggesting that *ost1* might be specific to ABA-dependent signaling.

The AAPK purified from guard cells of *Vicia faba* is likely to be an ortholog of OST1 (Li *et al.*, 2000). A point mutation created by site-directed mutagenesis



in the ATP-binding loop of the AAPK blocks, ABA induced stomatal closure in a transient assay. In response to ABA, the activated AAPK can phosphorylate a specific RNA-binding protein (AKIP) which then binds to a dehydrin transcript (Li *et al.*, 2002).

The rice genome also has 10 SnRK homologs responsive to hyperosmotic stress, and like *Arabidopsis*, three of them are also strongly stimulated by ABA (Kobayashi *et al.*, 2004). The wheat homolog PKABA1, identified on the basis of its induction in embryos, has been shown by transient expression assays to suppress GA-inducible promoters in response to the ABA signal (Gómez-Cadenas *et al.*, 2001) suggesting that PKABA1 may act in ABA–GA signal cross-talk during embryo development. One of its targets may be a bZIP-type transcription factor TaABF which was shown to interact with PKABA1 in the yeast two-hybrid system (Johnson *et al.*, 2002).

Certain members of the SnRK3 family act as global regulators of stresses, including those that are ABA dependent. The *SOS2* gene encoding SnRK3.11 was identified in a genetic screen for mutants overly sensitive to salt (Zhu *et al.*, 1998; Lui *et al.*, 2000). *SOS2* is activated by the binding of the calcium-binding protein *SOS3* in response to a cytosolic calcium signal triggered by the salt stress. As mentioned, *SOS2* also binds *ABI2* (Ohta *et al.*, 2003) suggesting that these two proteins act at the interface between salt and ABA signaling pathways. Similarly, *Arabidopsis* plants suppressed for the expression of the *PSK3/SnRK3.1* and *CIPK/SnRK3.17* genes were shown to be hypersensitive to ABA in seed germination (Guo *et al.*, 2002; Kim *et al.*, 2003). The mutant *psk3* also displays ABA-hypersensitive seedling growth, stomatal closure and expression of marker genes. Besides interacting with *ABI2* (and weakly with *ABI1*), *PSK3* has been recently shown to interact with and phosphorylate *in vitro* an AP2-containing transcriptional repressor *AtERF7* (Song *et al.*, 2005). Overexpression of *AtERF7* led to reduced sensitivity of guard cells to ABA; by contrast, experimentally reducing its expression by RNA interference caused hypersensitivity to ABA for germination. *AtERF7* was shown to mediate its suppressor function through the histone deacetylase *HDA19* and *AtSin13*, the latter of which is homologous to global repressors of transcription found in humans. Thus, signaling elements previously thought to belong to distinct response pathways seem to be components of a dynamic complex in the global regulation of hyperosmotic stresses (*AtHB6/SOS2/ABI2/ABI1/ SnRK3.1/AtERF7/AtSin13/ HD19*).

The SnRK1 family is comprised of only three members and their involvement in ABA signaling is more indirect. Two of these, *AKIN10* and *AKIN11*, can interact with a WD40-containing protein Pleiotropic Regulatory Locus 1 (*PRL1*) (Bhalerao *et al.*, 1999), an insertion mutant of which showed multiple defects including enhanced sensitivity to ABA (Németh *et al.*, 1998). More importantly, the AKIN immunocomplexes recovered from *prl1* plants showed higher kinase activity as compared to that from the wild type using a synthetic peptide as substrate. The pleiotropic phenotypes of *prl1* and higher SnRK-type kinase activity suggest that *PRL1* may regulate other components from a variety of other signaling pathways, including kinases from the SnRK2 and SnRK3 families.

## 1.6 ABA signaling in guard cells: simple movements controlled by complex mechanisms

ABA stimulates stomatal closure or inhibits its opening. The early signaling events in guard cells are parallel and entail the production of NAD(P)H-dependent reactive oxygen species (ROS), which stimulates hyperpolarization-activated  $\text{Ca}^{2+}$  permeable  $I_{\text{Ca}}$  channels. Moreover, ABA-induced elevation in cytoplasmic  $\text{Ca}^{2+}$  leads to activation of slow-anion channels and inactivation of the inward-rectifying  $\text{K}^+$  channel, which contributes to turgor reduction and stomatal closure (Hetherington, 2001; Schroeder *et al.*, 2001; Mäser *et al.*, 2003; Fan *et al.*, 2004; Roelfsema & Hedrich, 2005). ABA could also concomitantly suppress the activity of the plasma membrane  $\text{H}^+$ -ATPases (Zhang, X. *et al.*, 2004), which favors even more depolarization. Another pathway is the alkalinization of the guard cell cytosol, which directly enhances  $[\text{K}^+]_{\text{out}}$  channel activity (Blatt, 2000) and downregulates the transient R-type anion channels (Schulz-Lessdor *et al.*, 1996). The sustained efflux of both anions and  $\text{K}^+$  from guard cells via anion and  $[\text{K}^+]_{\text{out}}$  channels contribute to loss of turgor, leading to stomatal closing.

The above signaling scheme is derived from numerous experiments using guard cell protoplasts and epidermal peels as models, and strongly points to  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation stimulated by ABA as central in stomatal closure. ABA has also been reported to trigger repetitive  $[\text{Ca}^{2+}]$  oscillation (Staxén *et al.*, 1999; Allen *et al.*, 2000) and that the frequency of oscillation is signal specific (McAinsh & Hetherington, 1998; Allen *et al.*, 2001; Hetherington & Brownlee, 2004). However, with recent improvement in biophysical techniques, it is now possible to study guard cells within an intact plant (Roelfsema *et al.*, 2001). The expected increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  induced by ABA, however, was not observed when guard cells were measured *in situ*. Furthermore, guard cells possess a plasma membrane  $\text{Ca}^{2+}$  receptor, CAS. When expression of CAS was diminished by expression of antisense constructs, guard cells were no longer responsive to the rise of extracellular  $\text{Ca}^{2+}$  that triggers a rise in intracellular  $\text{Ca}^{2+}$  (Han *et al.*, 2003). These stomata, nonetheless, still responded to ABA. A basal level of  $\text{Ca}^{2+}$  is still required, however, as a chelating agent like 1,2-bis(2-aminophenoxy)ethane-N,N',N',-tetraacetic acid (BAPTA) can block stomatal closure by ABA (Levchenko *et al.*, 2005). It is further remarkable that many of the ABA signaling intermediates (inositol 1,4,5 trisphosphate, inositol-hexakisphosphate, nicotinic acid adenine dinucleotide phosphate and cyclic ADP-ribose) could no longer mimic ABA in activating anion channels in guard cells within intact plants (Levchenko *et al.*, 2005). It is still too early to reconcile these newer results with those obtained from guard cell protoplasts and epidermal peels. However, the ability of parallel signaling pathways to compensate for one another depending on the physiological state of the cell may lie at the heart of the problems in interpreting these results.

One striking aspect of the protein intermediates in ABA signals is the heterogeneity of their presumptive functions. These include kinases and protein phosphatases, phospholipases and G-proteins. Again only major advances in the past years will

be covered here, as complementary reviews are available (Fedoroff, 2002; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Fan *et al.*, 2004). *Arabidopsis* has a single gene each for the  $G_\alpha$ -subunit and  $G_\beta$ -subunit, and two genes for the  $G_\gamma$ -subunits. Recent studies of the *Arabidopsis* mutant *gpa1* disrupted for the  $\alpha$ -subunit of the heterotrimeric G-protein showed lack of ABA inhibition of stomatal opening and  $[K^+]_{in}$  activity (Wang, X.-Q. *et al.*, 2001; Fan *et al.*, 2004). However, *gpa1* also disrupts other hormone signals such as auxin (Ullah *et al.*, 2001) so this protein alone is unlikely to determine response specificity. GPA1 forms a complex with GCR1, the only G-protein-coupled receptor in *Arabidopsis* (Pandey & Assmann, 2004). The T-DNA mutant *gcr1* was shown to be more resistant to low humidity, and the overall stomatal movement is more sensitive to ABA suggesting that the receptor is a negative regulator of ABA sensitivity.

In animals, sphingosine-1-phosphate (S1P) signals through G-proteins and the phospholipid was discovered to increase  $[Ca^{2+}]_{cyt}$  and to stimulate stomatal closure in *Commelina communis* (Ng *et al.*, 2001). S1P is synthesized from the long-chain amine alcohol sphingosine by sphingosine kinase, and this activity in guard cells has been shown recently to be stimulated by ABA (Coursol *et al.*, 2003). Importantly, stimulation of stomatal closure or inhibition of stomatal opening by S1P is abolished in the *gpa1* mutant. Furthermore, patch-clamp analysis of *Arabidopsis* guard cells showed that S1P inhibits  $[K^+]_{in}$  channels, and this inhibition is attenuated in the *gpa1* mutant (Coursol *et al.*, 2003).

We have already mentioned that a series of elegant experiments have demonstrated that a specific RNA-binding protein AKIP in *Vicia faba* binds to a dehydrin mRNA in response to ABA (Li *et al.*, 2002). It would be interesting to identify the precise composition of the RNA cargo transported by AKIP in response to ABA, and their respective roles in guard cell or general responses to osmotic stress. Also, reverse genetics and genomics targeted to guard cells as a combined approach should accelerate our identification of elements inaccessible by classical genetics because of redundancy. Schroeder and colleagues (Leonhardt *et al.*, 2004) have shown that of the 8100 *Arabidopsis* genes represented on an Affymetrix oligo-based microarray, only 64 of them seem to be preferentially expressed in guard cells. The utility of this database to help identify guard cell-specific signaling components is already proven by the finding of a T-DNA mutant disrupted in an ABA-responsive PP2C gene encoding *AtPP2C-HA*, which showed ABA-hypersensitive.

## 1.7 ABA as antagonizing signal to light in stomatal movement

During the day, guard cells have to confront simultaneously sunlight which stimulates stomatal opening and the need to avert excessive transpiration by closing the stomatal pores. Light stimulates stomatal opening. Blue light (390–500 nm) turns out to be a very effective spectral component in stimulating stomatal opening (Iino *et al.*, 1985). It triggers, through an unknown cascade initiated by the receptors PHOT1 and PHOT2 (Kinoshita *et al.*, 2001; Sakamoto & Briggs, 2002), the phosphorylation of

the C-terminus of the  $H^+$ -ATPase to release the protein from autoinhibition, allowing access by 14-3-3 proteins to maintain the proton pump in the active conformation (Kinoshita & Shimazaki, 1999). ABA counteracts blue-light-dependent activity of the  $H^+$ -ATPase and locks the membrane potential to depolarized state, and allowing the continuous effluxes of cations and anions (Shimazaki *et al.*, 1986; Schroeder *et al.*, 2001). Recent evidence showed that ABA suppresses the hydrolytic activity of the  $H^+$ -ATPase, most likely by decreasing the phosphorylation of this proton pump, which in turn would have lower affinity for the binding of 14-3-3 proteins (Zhang, X. *et al.*, 2004). There is no firm idea concerning the types of kinases and protein phosphatases that ensure the correct balance of the  $H^+$ -ATPase activity. However, the global stress regulators such as the OST1 or related kinases (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002) and ABI1/2 PP2Cs (Leung *et al.*, 1997; Leonhardt *et al.*, 2004), which are expressed in guard cells, might also be interesting to explore as potential regulators of light-ABA cross-talk.

Two R2R3 MYB transcription factors expressed in guard cells seem to have important roles in the light response pathways (Cominelli *et al.*, 2005; Liang *et al.*, 2005). The *atmyb60* mutant is impaired in light-induced stomatal opening (Cominelli *et al.*, 2005), while the other, *atmyb61* has increased stomatal conductance (Liang *et al.*, 2005). Expression of the *ATMYB60* seems to be independent of the *PHOT* pathways, but the transcript is stimulated by ABA and blocked by the *abi1-1* mutation. However, *atmyb60* itself did not display altered responsiveness to the applied hormone (Cominelli *et al.*, 2005). Wild-type ABA sensitivity was similarly found for *atmyb61*. Thus, while these two R2R3 MYB proteins represent the first examples of transcription factors in controlling light-regulated stomatal responses, how they are related to the ABA and blue-light signaling networks awaits to be further explored.

## 1.8 Concluding remarks

Major progress has now been made in elucidating the pathways for ABA synthesis and degradation. As ABA is a mobile signal, the use of *Arabidopsis* as a genetic and molecular model has led to rapid advances toward understanding of how and where this hormone is produced. Current evidence indicates that vascular bundles are a major site of hormone synthesis, but the kind of molecular mechanisms that regulate ABA distribution to other parts of the plants during normal growth and during stress response remain to be determined. Another challenge will be to unravel the signaling pathways regulating ABA levels. The availability of both promoter sequences from cloned genes or developing new inhibitors of metabolic enzymes could afford new approaches, such as reporter or chemical genetic screens, in order to isolate specific transcription factors and novel signaling mutants. Several transcription factors and their corresponding *cis*-acting sequences in target promoters have already been studied in detail with respect to ABA signaling. A striking feature is

that these transcription factors are subject to, at least in part, controlled proteolysis. Perhaps the initial steps in ABA action in seeds have parallels with auxin signaling in that some of the proteolytic components in ABA pathways may also bind the hormone directly.

Unexpectedly, the *Arabidopsis* RNA-binding protein FCA was shown to be a nuclear ABA receptor, revealing a direct but subtle role of ABA in the autonomous flowering pathway. The binding motif(s) of ABA remains to be defined in the ABAP1 and the FCA proteins. This may provide one important hint into uncovering other furtive ABA receptors. As RNA metabolism appears to be an essential mechanism in ABA signaling, and half of the nearly 200 RNA-binding proteins with either RMM and KH homology domains in the *Arabidopsis* are plant specific, some of these may also be involved in ABA perception.

The importance of the various second messengers in guard cell responses to ABA remains unsettled. Recent technical improvements permitting measurements of guard cells to be made within intact plants suggest that many of these intermediates seem dispensable for the ABA responses observed in protoplasts or in epidermal peels. Perhaps the effects of mobile apoplastic messages ( $H^+$  or other small solutes) from neighboring cells have subtle but non-negligible influence on guard cell responses. These solutes may affect cell wall enzymes of which some may be involved in signaling (Fan *et al.*, 2004). Guard cell signaling in relationship to drought has become tractable genetically in the last few years (Merlot *et al.*, 2002), and it might be fruitful to reassess the importance of these second messengers by combining with mutant analysis, like the work on SIP and G-proteins (Coursol *et al.*, 2003).

The overall heterogeneity in the cast of protein intermediates makes formulating a coherent ABA signaling model a daunting task. Probably, the "heterogeneity" reflects the different mechanisms in the diverse tissues that have not been taken into account in our conceptual model. At present, mutations in the SnRK, PP2C and RNA-binding proteins seem more prevalent than other classes. In particular, SnRKs and the PP2C ABI1 and ABI2 appear to form the core of a dynamic complex involved in the global regulation of osmotic stress. The next step toward understanding ABA action will likely take advantage of the available SnRKs, PP2Cs and RNA-binding proteins as starting points to identify their physiological substrates and entire signaling complexes by proteomic approaches.

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## 2 Auxin metabolism and signaling

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### 2.1 Introduction

Auxin responses in plants were first observed and described in the 19th century (reviewed in Slovin *et al.*, 1999). Elegant but simple experiments by early investigators indicated that a system for mobile signaling existed that allowed plants to respond to environmental signals such as light and gravity distant from their site of perception. While the 20th century saw great progress in studies of auxin chemistry and physiology, we nevertheless approached the 21st century with many unanswered questions about how plants perceive and respond to auxin, how auxin is made within the plant, and how such processes are themselves regulated. In this review, we will focus on these central questions and the impressive progress that has been made to illuminate those areas that have remained perplexing issues for a very long time.

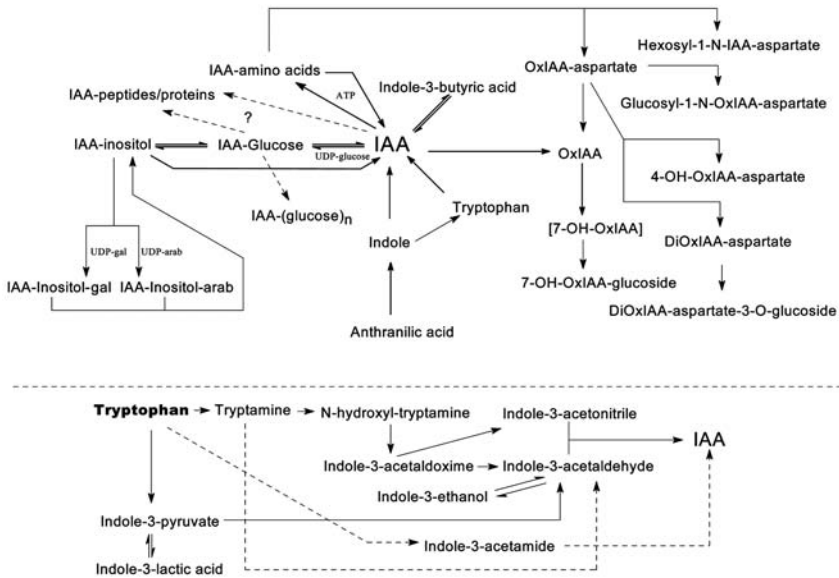
### 2.2 Auxin metabolism

#### 2.2.1 Indole-3-acetic acid biosynthesis

For the past 60 years, research on indole-3-acetic acid (IAA) biosynthesis in plants (Fig. 2.1) has focused on the degradation of the amino acid tryptophan by sequential oxidative deamination and decarboxylation (Wildman *et al.*, 1946). Although there were questions raised, the basic concept remained relatively unchallenged until the early 1990s when work on several systems showed that IAA biosynthesis occurs via two separate pathways (Woodward & Bartel, 2005). The metabolism of IAA plays an important part in the growth and development of plants, from embryogenesis (Ribnicky *et al.*, 2002) through fruit ripening (Cohen, 1996) and senescence (Quirino *et al.*, 1999). Fundamental reassessments have occurred regarding the metabolic processes involved in making indolic auxins and consequently our ideas concerning the complexity of the metabolic control of these regulatory hormones have changed (Bartel, 1997; Normanly & Bartel, 1999).

##### 2.2.1.1 The tryptophan-independent pathway

One of the first uses of stable isotope methods to track IAA biosynthesis by mass spectrometry (MS) illustrated the advantages of such methods. Baldi *et al.* (1991) compared normal plants to an IAA-overproducing mutant, jsR<sub>1</sub>, of *Lemna gibba* in an effort to examine the theory that D-, not L-tryptophan was the closer precursor to IAA. These experiments indicated that while [<sup>15</sup>N]D-tryptophan was ineffective,



**Figure 2.1** IAA metabolism. Summary of the metabolic reactions involved in IAA metabolism (upper) and in tryptophan-dependent IAA biosynthesis (lower) discussed in the text. Solid lines indicate reactions for which there is substantial evidence for *in vitro* activity, while dashed lines indicate reactions for which somewhat less evidence has been published. Not all reactions or compounds are found in all plants. The majority of the reactions presented are from studies of *Arabidopsis* and maize. A more detailed analysis of specific reactions of IAA biosynthesis, conjugation and oxidation routes can be found in Normanly *et al.* (2004) and in Woodward and Bartel (2005).

[<sup>15</sup>N]L-tryptophan was also converted at relatively slow rates, suggesting that the widely held views about IAA biosynthesis from tryptophan needed a critical reevaluation. Rapid kinetic studies have shown that, even in *Lemna gibba*, L-tryptophan labeling overestimates the contribution of tryptophan as an auxin precursor since exogenous tryptophan was preferentially used to label IAA as compared to tryptophan synthesized *in vivo* (Rapparini *et al.*, 1999).

The only known non-conditional tryptophan auxotroph, *orp* (orange pericarp), proved to be a uniquely suitable genetic system with which to reevaluate the tryptophan precursor hypothesis (Wright *et al.*, 1991). *orp* mutants completely lack tryptophan synthase β-activity due to mutations in two unlinked loci, *orp1* and *orp2* (Wright *et al.*, 1992). When grown on media containing 30% [2H<sub>2</sub>]O or [<sup>15</sup>N]anthranilate, only the wild-type seedlings showed significant incorporation of label into tryptophan, while both wild-type and mutant seedlings showed substantial incorporation into IAA. Thus, in *orp* seedlings, *de novo* IAA biosynthesis occurs without tryptophan as a precursor.

A similar series of studies using *Arabidopsis* conditional tryptophan auxotrophs (Last and Fink, 1988) extended the results from maize (Normanly *et al.*, 1993). These studies focused on three mutants, *trp1-100*, *trp2*, and *trp3*, which are deficient in anthranilatephosphoribosyl transferase, tryptophan synthase β, and tryptophan

synthase  $\beta$  activities, respectively (Last *et al.*, 1992). The tryptophan synthase  $\beta$ -mutant showed elevated levels of IAA (Normanly *et al.*, 1993). The other two mutants had normal levels of IAA. Based on  $[^2\text{H}_2]\text{O}$  labeling, all three mutants were synthesizing IAA *de novo*. These results suggest that the branch point for IAA biosynthesis from the pathway to tryptophan is between tryptophan synthase  $\alpha$  and  $\beta$ . Ouyang *et al.* (2000), using an antisense approach to lower the levels of indole-3-glycerolphosphate (IGP) synthase activity in plants, showed a decrease in IAA levels and a low auxin phenotype. The authors concluded that IGP was the likely precursor to IAA, however the results are subject to some interpretation since the conversion of IGP to indole is reversible. An unassociated tryptophan synthase  $\alpha$ -like gene appears to be present in *Arabidopsis* based on sequence analysis (Ouyang *et al.*, 2000), and the encoded protein could account for the production of free indole, as has been shown for the maize tryptophan synthase  $\alpha$ -like genes, *IGL* and *BX1* (Gierl & Frey, 2001).

Another model system used for studies of IAA biosynthesis is carrot cell cultures, which can be induced to form somatic embryos by removal of auxin (i.e. 2,4-D) from the medium. In suspension cultures, L-tryptophan can be converted to IAA (Michalczyk *et al.*, 1992). However, when these same cells are induced to undergo embryogenesis, the tryptophan-independent pathway predominates. Carrot suspension cell cultures grown in the presence or absence of the synthetic auxin 2,4-D incorporated label from  $[^2\text{H}_2]\text{O}$  into IAA, indicating that *de novo* biosynthesis was occurring. Cells grown on media-containing  $[^{15}\text{N}]\text{indole}$ , both with and without 2,4-D, gave the same result. However, when cells were labeled with  $[^2\text{H}_5]\text{L-tryptophan}$ , only the cultures grown in the presence of 2,4-D incorporated the ring deuterium into IAA. In all cases, with or without 2,4-D, tryptophan pools were equally labeled. Thus, carrot cells make IAA by two different pathways at different developmental stages, because label incorporation into IAA from tryptophan changed without a change occurring for label incorporation from the earlier precursors, indole or  $[^2\text{H}_2]\text{O}$ . Subsequent studies have confirmed both developmental and environmental regulation of IAA biosynthetic pathways, as determined by changes in labeling patterns, in several plant species (Epstein *et al.*, 2002; Rapparini *et al.*, 2002; Sztein *et al.*, 2002) and in an evolutionary context (Sztein *et al.*, 2000; Cooke *et al.*, 2004).

IAA biosynthesis was studied by several laboratories using *in vitro* enzymes prepared from maize tissues. Rekoslavskaya and Bandurski (1994) showed that extracts from liquid endosperm tissue converted indole to IAA, suggesting that the tryptophan-independent pathway might contribute to the newly synthesized IAA. However, extensive tryptophan to IAA conversion was also detected (Rekoslavskaya, 1995).

Östin *et al.* (1999) described an *in vitro* enzymatic system from maize seedlings in which  $[^{14}\text{C}]\text{indole}$  was converted into  $[^{14}\text{C}]\text{IAA}$ . Biosynthesis was shown to be independent of tryptophan in this system because:

- (1)  $[^{14}\text{C}]\text{tryptophan}$  was not converted to  $[^{14}\text{C}]\text{IAA}$
- (2) The addition of cold tryptophan did not inhibit conversion of  $[^{14}\text{C}]\text{indole}$  to  $[^{14}\text{C}]\text{IAA}$

- (3) Serine did not enhance the same conversion
- (4) Addition of non-labeled indole decreased the formation of [ $^{14}\text{C}$ ]IAA.

IAA biosynthesis was also shown to occur in an *in vitro* system derived from extracts of *orp* maize seedlings (Östin *et al.*, 1999; Ilic & Cohen, 2004), which lack a functional tryptophan synthase  $\beta$ .

#### 2.2.1.2 IAA biosynthesis from tryptophan

The combined techniques of stable isotope labeling and gas chromatography-mass spectrometric (GC-MS) analysis of isotopic enrichment of suspected intermediates provide more data and allow evaluation of labeling patterns in detail not previously possible (Cooney & Nonhebel, 1991; Wright *et al.*, 1991; Michalczuk *et al.*, 1992; Normanly *et al.*, 1993; Östin *et al.*, 1999). Thus, it is now feasible to obtain a more accurate assessment of precursor-product relationships. Stable isotope techniques, however, are also subject to error if multiple pools of compounds exist within the cell and if only specific pools are involved in the biochemical process being studied (Cooney & Nonhebel, 1991; Sitbon *et al.*, 1993; Rapparini *et al.*, 1999). Some direct experimental analysis of the impact of these problems has shown that although multiple pools of tryptophan exist in carrot cell cultures, both pools can be labeled following isotopic tryptophan application (Michalczuk *et al.*, 1992). Time, as well as pool localization, can be an issue. Bialek *et al.* (1992) showed that tryptophan conversion to IAA accounted for essentially all of the IAA production in germinating axes of bean when label incorporation was analyzed after several days of labeling following the surgical removal of the cotyledons. However, a decade later, using methods allowing shorter labeling times, this experimental system was revisited in a series of studies (Sztejn *et al.*, 2002) where it was shown that tryptophan conversion was only operative in the first hours after cotyledon removal, followed by several days when the tryptophan-independent pathway predominated, suggesting wound activation of tryptophan to IAA conversion.

Compelling evidence from precursor and deuterium oxide labeling experiments indicate that intact etiolated maize seedlings do not produce significant IAA from *de novo* biosynthesis (Pengelly & Bandurski, 1983; Jensen & Bandurski, 1996), which is likely a result of exceptional biosynthetic activity of the endosperm during seed development (Jensen & Bandurski, 1994). Maize liquid endosperm *in vitro* extracts are capable of converting radioactive indole to both IAA and tryptophan (Rekoslavskaya & Bandurski, 1994). Most remarkably, IAA accounted for up to 60% of the product and, although initial results suggested that tryptophan might not be directly involved, subsequent experiments showed that most of the labeled indole actually was converted to tryptophan before going onto IAA (Rekoslavskaya, 1995; Ilic *et al.*, 1999; see also Glawischnig *et al.*, 2000). IAA biosynthesis in this system has since been shown to be sensitive to oxidation potential (Ilic *et al.*, 1999), which is unexpected because the previously described routes from tryptophan are oxidative or simple transferase reactions. In addition, tryptamine, indole-3-acetamide, indole-3-acetonitrile, and indole-acetaldoxime (IAOx) were shown to be not directly involved in the reaction, while a

peptide-bound tryptophan intermediate was isolated (Hendrickson *et al.*, 2004). This experimental system, where relatively large rates of IAA biosynthesis occur, clearly shows promise for understanding at least one route for IAA biosynthesis.

The relative contribution of the tryptophan-dependent biosynthesis pathway in *Arabidopsis thaliana* has been examined critically (Mueller *et al.*, 1998; Mueller & Weiler, 2000), however experimental levels of tryptophan and indoleacetonitrile (IAN) used were 15–25 $\times$  of their endogenous levels (Tam *et al.*, 1995; Ilic *et al.*, 1996) yielding results difficult to interpret. Similarly, Park *et al.* (2003) took a high substrate approach with maize activities converting IAN (a possible intermediate in the tryptophan-dependent pathway) where they reported assays in the millimolar range, although the reported IAN endogenous concentrations were lower orders of magnitude.

Redundant pathways have been defined within the tryptophan-dependent pathway by using gain-of-function methods in *Arabidopsis* (Cohen *et al.*, 2003). Activation tagging identified the *YUCCA* gene that encodes a novel flavin monooxygenase catalyzing the rate-limiting N-hydroxylation of tryptophan to create N-hydroxyl tryptamine (Zhao *et al.*, 2001). This defined a new pathway not formerly predicted for IAA biosynthesis from tryptophan. Although at least two paralogs are present in *Arabidopsis*, the *YUCCA*-like flavin monooxygenase from petunia is apparently a single-copy gene (Tobena-Santamaria *et al.*, 2002).

Recent advances concerning the genetics of secondary product metabolism have resulted in increased focus on the role of cytochrome P450s in the reactions leading to indolic glucosinolates (Bak & Feyereisen, 2001; Bak *et al.*, 2001) and has resulted in important advances in our knowledge of IAA biosynthesis as well. For example, overexpression of an *Arabidopsis* gene encoding the cytochrome P450, CYP83B1/*SUPERROOT 2* (*SUR2*; Barlier *et al.*, 2000), or overexpression of a related cytochrome P450, CYP83A1, both resulted in increased indolic glucosinolate levels and a morphologic phenotype consistent with underproduction of IAA. Conversely, *sur2* mutants exhibit increased adventitious rooting and epinasty, consistent with IAA overproduction. It has been suggested that CYP83B1 serves as a gatekeeper between IAA and indolic glucosinolate biosynthesis, but downstream from the most likely branch point from IAA production where IAOx is an intermediate (Celenza, 2001).

The conversion of tryptophan to IAOx is catalyzed by two other cytochrome P450s, CYP79B2 and CYP79B3 in *Arabidopsis*, but whether IAA or only glucosinolates were produced from IAOx was not clear (Hull *et al.*, 2000). The CYP83B1/CYP83B2 results pointed to the most likely role for these cytochrome P450s being to supply IAOx for conversion to 1-*aci*-nitro-2-indolyl-ethane, the committed step of indolic glucosinolate biosynthesis. Analysis of CYP79B2 and CYP79B3 function as it relates to *YUCCA* function, however, puts these two proteins squarely in the middle of another pathway utilizing IAOx in *Arabidopsis* for the production of IAA from tryptophan (Zhao *et al.*, 2002).

The complexity of understanding IAA biosynthesis appears to be well illustrated in *Arabidopsis*, where several potential pathways compete for attention of the research

community. It appears likely that several pathways are present, perhaps in different cells, developmental stages, or as regulated by environmental inputs. In addition to the pathways involving monooxygenases and/or cytochrome P450s, reports of the presence of indole-3-pyruvic acid (Tam & Normanly, 1998) and indole-3-acetamide (Pollmann *et al.*, 2002) as well as the amidase, a multienzyme complex (Mueller & Weiler, 2000; Pollmann *et al.*, 2003), and nitrilases (Normanly *et al.*, 1997) suggest that the potential for many unique routes to IAA remains a very real possibility.

### 2.2.2 IAA conjugates in plants

All plants and plant parts so far examined contain most of their IAA in conjugated form and only minute amounts of the phytohormone as the free acid. Some of the IAA conjugates that have been identified are esters or acyl anhydrides with simple sugars or cyclitols, larger-molecular-weight polysaccharides, or the carbohydrate component of glycoproteins. IAA can also be conjugated by amide linkage to amino acids, such as aspartate, or to peptides or proteins.

#### 2.2.2.1 IAA-peptide conjugates

The known higher-molecular-weight amide conjugates consist of a hydrophobic 3.6 kDa peptide (Bialek & Cohen, 1986), a 35 kDa protein (Walz *et al.*, 2002) from bean and several immunologically related proteins of higher-molecular-weight from *Arabidopsis* and several other species (Dunlap *et al.*, 1996; Walz *et al.*, 2002), and a class of maize zein protein (Leverone *et al.*, 1991).

Bialek and Cohen (1986) isolated a compound with very low mobility on thin-layer chromatography (TLC), stained positive for indole compounds, and was subsequently isolated from bean seeds and found to yield IAA upon hydrolysis in 7 N NaOH at 100°C. The properties of this compound were quite different from those of simple IAA–amino acid conjugates and it proved to be a conjugate consisting of two moieties of IAA attached to a 3.6 kDa peptide.

Several early studies had indicated that protein-like compounds were formed by plants from supplied radioactive IAA. More recently, such methods have shown that IAA–proteins are formed based on [<sup>14</sup>C]-IAA feeding experiments with melon tissue (Dunlap *et al.*, 1996). Antibodies against the 3.6 kDa IAA–peptide from bean (Cohen *et al.*, 1988) localized immunoreactive materials in immature melon fruit (Cohen *et al.*, 1995) to the vascular tissue and epidermal layers, consistent with mass spectral measurements of amide IAA localization (Dunlap *et al.*, 1996).

In bean, amide conjugated IAA does not begin to accumulate in significant amounts until late in seed development (Bialek *et al.*, 1992). By the time the seed is mature, however, most of the total IAA is present as protein conjugates. During seed germination the protein conjugates, after an initial rapid decline, began to increase in amount again early in seedling growth (Bialek & Cohen, 1992). The content of free IAA as well as the content of amide IAA in the embryonic axes began to increase as soon as the growth of the axes started, and the level of IAA conjugates in the whole seed remained relatively high even after a week of growth.



The gene for the major bean IAA protein (*iap1*) was isolated and cloned from bush bean (*Phaseolus vulgaris*) seeds. Walz *et al.* (2002) showed that the 957 bp sequence encoded a 35 kDa protein and their immunologic and analytical data suggested that auxin modification of a small class of proteins may be a feature common to many plants. Quantitative GC-MS analysis of the purified IAP1 showed a protein to IAA ratio of 2:1.

Previous studies showed that *Arabidopsis* has both ester and amide conjugated IAA (Tam *et al.*, 2000); however, in total the low-molecular-weight conjugates identified did not account for the bulk of the conjugate pool. Immunostaining of different *Arabidopsis* tissues showed that putative IAA-proteins were localized to the root meristem and outer cell regions of the cotyledons and radical of *Arabidopsis* seeds (Walz *et al.*, 2002). *Arabidopsis* seed IAA-proteins were partially purified and subjected to alkaline hydrolysis. GC-MS analysis confirmed the presence of IAA covalently bound to protein. These results established the presence of IAA-proteins in dicots other than bean (reviewed in Ljung *et al.*, 2002) and confirmed that the major IAA conjugates in *Arabidopsis* are peptides and proteins.

Leverone *et al.* (1991) showed that IAA-peptide formation might be more widespread among plants by finding that the maize storage protein zein contained amide bound IAA. In this case, however, IAA was present in only a very minor fraction of the total seed zein (1 molecule IAA to 175 molecules of zein), thus it is possible that a discrete class of zein peptides is conjugated.

#### 2.2.2.2 Amino acid conjugates

In most higher and many lower plants, applied IAA is rapidly conjugated to form IAA-aspartate (reviewed in Cohen & Bandurski, 1982). The formation of IAA conjugates is widely believed to be a means of removal of excess IAA produced during certain times of plant development, and in mutant plants where indolic precursors accumulate (Wright *et al.*, 1991). The ability of plant tissues to make IAA-aspartate, as well as aspartate conjugates of a variety of synthetic auxins, is induced by pretreatment with active auxins (Venis, 1972), and this induction is blocked by inhibitors of RNA and protein synthesis. After almost 50 years of study, recently an *in vitro* system from plants has been described that accounts for the formation of IAA-amide conjugates (Staswick *et al.*, 2005) via a mechanism where the acid auxin is adenylated followed by acyl transfer to the amino acid. The actual gene for this reaction had been discovered almost 20 years previously, when *GH3* from soybean was shown to be rapidly induced following auxin treatment (Hagen & Guilfoyle, 2002), however the biochemical activity of the gene product remained undefined. A number of different IAA-amino acid conjugates (e.g. IAA-aspartate, -glutamate, -alanine) have been identified in plants and plant cell cultures (Slovin *et al.*, 1999; Ljung *et al.*, 2002), and it appears that several of these could be formed by similar mechanisms.

#### 2.2.2.3 Amide conjugate hydrolysis

Auxin conjugates can be used as "slow release" forms of IAA in plant tissue cultures, probably because of their slow hydrolysis in plant cells (Magnus *et al.*, 1992).

*In vitro* hydrolysis was shown by Bialek *et al.* (1983) for specific applied conjugates, and Ludwig-Muller *et al.* (1996) showed that a specific conjugate hydrolase was induced in Chinese cabbage upon infection with *Plasmodiophora brassicae*.

A genetic approach to the analysis of IAA conjugate hydrolysis in plants resulted in the first identification of a gene (*ILR1*) coding for a hydrolase of IAA conjugates (Bartel & Fink, 1995; Bartel, 1997). This enzyme preferentially hydrolyzes IAA-leucine and IAA-phenylalanine. *ILR1* is representative of a gene family whose members exhibit varying substrate specificities (Davies *et al.*, 1999). *IAR3*, for example, shows a high level of specificity for IAA-L-alanine while *ILL2* shows the highest *in vitro* activity and is also the most promiscuous in its substrate requirements (LeClere *et al.*, 2002). *ILR1* and *IAR3* are found predominately in roots, while *ILL2* is predominately found in shoots (Rampey *et al.*, 2004). Triple mutant (*ilr1*, *iar3*, and *ill2*) analysis has shown that lateral shoot number, hypocotyl elongation, auxin sensitivity and IAA levels are altered, suggesting that conjugate hydrolysis is involved in regulation of such processes *in planta* (Rampey *et al.*, 2004).

Enzymes from bacterial sources that are capable of highly selective hydrolysis of IAA-amino acids have been reported. For example, an IAA-L-aspartate hydrolase was purified (Chou *et al.*, 1996) and subsequently cloned. Additional characterization at the level of the gene and analysis of the enzyme protein revealed that it is related to other bacterial amidohydrolases, shows homology to the *ILR1* gene of *Arabidopsis* (Chou *et al.*, 1998), and transformation into *Arabidopsis* shows a mild high auxin phenotype (Tam *et al.*, 2000). Subsequent analysis revealed that two zinc-binding histidine residues conserved in bacterial and plant enzymes, His-404 and His-405, were critical for determination of substrate specificity and activity, respectively (Chou *et al.*, 2004). A bacterial enzyme with specificity for IAA-L-alanine was recently reported from *Arthrobacter ilicis* and related bacterial strains (Chou & Huang, 2005).

#### 2.2.2.4 Ester conjugates

The ester conjugates of maize endosperm have been extensively reviewed (Cohen & Bandurski, 1982). The higher-molecular-weight ester compound is a cellulosic glucan and the lower-molecular-weight compounds include IAA-glucose, IAA-*myo*-inositol and IAA-inositol glycosides. IAA-*myo*-inositol esters have also been found in rice (Hall, 1980) and horse chestnut (Domagalski *et al.*, 1987). Horse chestnut was also shown to contain the esters IAA-rutinose (IAA-glucosyl-rhamnose) and an IAA-desoxyaminohexose (Domagalski *et al.*, 1987).

The *in vitro* synthesis of 1-*O*- $\beta$ -D-IAA-glucose from IAA and uridine diphosphate (UDP)-glucose has since been described (Michalczyk & Bandurski, 1982; Leznicki & Bandurski, 1988a & b) and the full array of conjugate-synthesizing reactions of *Zea mays* have also now been studied *in vitro* (Bandurski & Nonhebel, 1984; Kowalczyk & Bandurski, 1990, 1991; Bandurski *et al.*, 1992). In maize, IAA-glucose is transacylated to *myo*-inositol to form IAA-*myo*-inositol (Bandurski & Nonhebel, 1984; Kesy & Bandurski, 1990). IAA-inositol may then be glycosylated to form IAA-*myo*-inositol-galactoside or IAA-*myo*-inositol-arabinoside by reaction with the appropriate uridine diphosphosugar (Bandurski & Nonhebel, 1984).



The enzyme catalyzing the synthesis of 1-*O*-IAA-glucose, IAA-glucose synthetase, was purified to homogeneity, characterized and cloned (Leznicki & Bandurski, 1988a & b; Szerszen *et al.*, 1994). Kowalczyk *et al.* (2002) showed that the enzymatic activity and protein levels of IAA-glucose synthase are increased following auxin treatment of maize coleoptiles. The reaction catalyzed by IAA-glucose synthase,



yields a product in which the acyl alkyl acetal bond between IAA and the aldehydic oxygen of glucose is approximately 1.4 kcal above that of the bond between glucose and UDP (Leznicki & Bandurski, 1988b). This energetically unfavorable reaction is “pulled” in the direction of conjugate formation by the energetically favored transacylation of the IAA moiety (Leznicki & Bandurski, 1988b; Szerszen *et al.*, 1994).

Isolation of the *Arabidopsis* gene encoding an IAA-glucose forming enzyme (Jackson *et al.*, 2001) as well as the availability of the maize gene has resulted in several groups generating transgenic plants with elevated and/or reduced capacity for IAA-glucose formation. As the reaction leading to the formation of IAA-glucose is dependent on factors in addition to the level of active enzyme, the biochemical basis for the phenotypes obtained with the transgenic plants is not easy to interpret. The effect of the transgene likely depends on the availability of both excess UDP-glucose and a suitable transacylation receptor in the transformed plant. While dicots as well as monocots form IAA-glucose (Cohen & Bandurski, 1982; Tam *et al.*, 2000; Jackson *et al.*, 2001) and some dicots contain IAA-*myo*-inositol (Domagalski *et al.*, 1987), the potential IAA-acyl acceptor in most dicots remains unknown. The potential does exist for amino acids or other amines to serve as an alternative substrate for such transacylations from IAA-glucose or, as recently proposed (Jakubowska & Kowalczyk, 2004), IAA-peptides or IAA-proteins could result from such a acyl transfer mechanism. Although proposed, such reactions have never been demonstrated *in vivo* and are proposed based on the concept that the constitutive route to IAA-amides would differ from the induced mechanism via the GH3 adenylation reaction (Staswick *et al.*, 2005). Antisense tomato seedlings showed reduced levels of IAA-glucose and in ester IAA pools, but no corresponding alteration in the levels of amide conjugates (Iyer *et al.*, 2005), suggesting that ester and amide conjugation are not linked via a common acyl transfer intermediate of IAA-glucose, and that amino acids may not, in this instance, serve as suitable indole-acyl acceptors. Surprisingly, levels of free IAA either are unaffected by the level of expression of enzymes for IAA-glucose formation (Ludwig-Mueller *et al.*, 2005) or increase or decrease in parallel with IAA-glucose changes (Jackson *et al.*, 2001; Iyer *et al.*, 2005).

Kowalczyk and Bandurski (1990, 1991) and Jakubowska and Kowalczyk (2005) reported on the co-fractionation of IAA-glucose synthetase and two enzymes for IAA-glucose hydrolysis, a 1-*O*-IAA-glucose hydrolase and a 6-*O*-IAA-glucose hydrolase, and suggested that these enzymes exist as a hormone-metabolizing complex. Kowalczyk *et al.* (2003) described a bifunctional indole-3-acetyl transferase that catalyzes synthesis and hydrolysis of indole-3-acetyl-*myo*-inositol in immature

endosperm of *Zea mays*. These studies may indicate that at least some aspects of IAA ester metabolism occurs via enzyme complexes.

### 2.2.3 IAA degradation

Ideas concerning IAA catabolism have also undergone substantial revision. IAA catabolism was thought to occur primarily through the action of IAA oxidase, a companion activity to most peroxidases of plant origin (reviewed in Normanly *et al.*, 1995). It is likely that the contribution of the decarboxylation pathway has been overestimated when studying IAA oxidation in homogenates or with cut tissue pieces. The concept of an "IAA oxidase" has received several serious challenges and this has been extensively reviewed (Normanly *et al.*, 1995; Slovin *et al.*, 1999).

In maize, oxidation of IAA *in vivo* yields oxindole-3-acetic acid (oxIAA) (Reinecke & Bandurski, 1983; Nonhebel, 1986). The general process of ring oxidation followed by glycosylation appears to be a general theme, although variable in its specific details in different species (reviewed in Slovin *et al.*, 1999; Normanly *et al.*, 2004).

The discovery that the IAA conjugate, IAA-aspartate, can be either hydrolyzed to yield free IAA (Bialek *et al.*, 1983) or serve as an entry point into IAA catabolism (Tuominen *et al.*, 1994) placed new emphasis on understanding the biochemistry of this branch point compound (reviewed in Normanly *et al.*, 2004). Plüss *et al.* (1989) found IAA-aspartate and oxIAA-aspartate were the major metabolites present after feeding IAA to *Populus tremula*. At least three different pathways for the direct oxidation of this IAA conjugate in different plant species are known (Normanly *et al.*, 2004). Thus, IAA-aspartate has a significant role in IAA degradation, and these findings demonstrate that links exist between the processes of conjugation and degradation.

## 2.3 Auxin signaling

The application of auxin to plant tissues elicits a multitude of effects including electrophysiologic and transcriptional responses, and changes in cell division, cell expansion, and cell differentiation. A central question in plant biology has long been how this simple molecule controls this staggeringly diverse array of responses. While this question is far from being answered, recent years have witnessed dramatic advances in our understanding of the molecular events underlying auxin action.

### 2.3.1 Auxin-responsive genes

Early molecular approaches to elucidate auxin action revealed that the hormone induces rapid and specific changes in gene expression and identified several auxin-responsive genes. Among the most thoroughly characterized are three gene families (*SAURs*, *GH3s*, and *Aux/IAAs*), which are rapidly induced in response to an auxin stimulus.

The S M A L L A X I N - U P R N As (*SAURs*) were initially identified in soybean as rapidly accumulating transcripts following a short auxin treatment (McClure &

Guilfoyle, 1987). *SAUR* genes have subsequently been identified in many other plants, including 77 predicted *SAURs* in the *Arabidopsis* genome. Several *SAUR* transcripts are highly unstable due to a conserved downstream element (DST) found in the 3' untranslated region (Newman *et al.*, 1993). These transcripts encode small proteins of 9–15 kDa, however sequence analysis provides few clues as to their function. Maize ZmSAUR2 has been shown to be a short-lived nuclear protein (Knauss *et al.*, 2003), and both ZmSAUR2 and ZmSAUR1 have been found to bind calmodulin *in vitro* in a calcium-dependent fashion (Yang & Poovaiah, 2000; Knauss *et al.*, 2003). What role, if any, the *SAURs* play in auxin signaling however remains to be established.

*Arabidopsis* contains 19 *GH3* genes, several of which are auxin inducible. Recent findings demonstrate that at least some *GH3* family members encode a novel class of acyl-adenylate-forming enzymes capable of acting as IAA–amino synthases in the conjugation of IAA to amino acids (Staswick *et al.*, 2005). This suggests that the auxin induction of *GH3* genes acts as a feedback mechanism that attenuates the auxin signal by inactivating IAA via conjugation. Consistent with this possibility, *Arabidopsis* seedlings overexpressing *GH3.6* exhibit resistance to applied IAA, while seedlings containing mutations in *GH3.5* or *GH3.17* exhibit increased sensitivity (Staswick *et al.*, 2005). It should be noted that not all *GH3* genes are auxin inducible, nor do all *GH3* proteins exhibit IAA–amino synthase activity. For example, *GH3.11/JAR1* is not transcriptionally regulated by auxin and has instead been shown to function as a jasmonic acid–amino synthase (Staswick & Tiryaki, 2004).

The *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes comprise the third large class of auxin-inducible transcripts. Originally identified from soybean and pea, *Aux/IAA* genes have subsequently been identified in many plants, including *Arabidopsis*, which contains a 29-member gene family (Liscum & Reed, 2002). As is the case for auxin induction of the *SAUR* genes, *Aux/IAA* induction occurs within minutes of auxin treatment and does not require new protein synthesis. The extent of induction, as well as the kinetics of expression varies considerably between different *Aux/IAA* family members (Abel *et al.*, 1995).

The *Aux/IAA* genes encode 20–35 kDa proteins that localize to the nucleus in all cases examined to date. Several lines of genetic and molecular evidence indicate that these proteins function as negatively acting transcription factors that repress auxin response. Albeit a few exceptions, *Aux/IAA*–proteins share four highly conserved motifs termed domains I, II, III, and IV. The C-terminal domains III and IV mediate dimerization with other *Aux/IAA*–proteins and with *AUXIN RESPONSE FACTORS* (ARFs), which also share these two motifs. Domain I functions as a putative transcriptional repression motif, and domain II constitutes a degron that targets the *Aux/IAA*–proteins for degradation by the ubiquitin–26S proteasome proteolytic pathway. The function of these highly conserved motifs and the role of *Aux/IAA*–proteins in auxin response are discussed in more detail below.

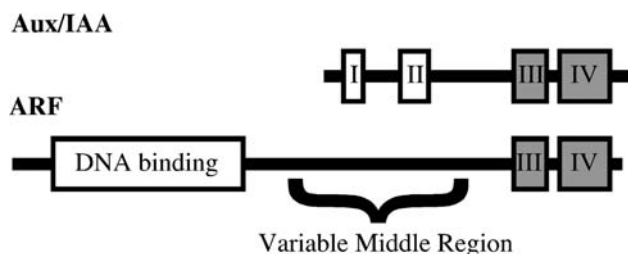
### 2.3.2 Auxin response factors

The upstream regulatory regions of several auxin-responsive genes contain one or more copies of the consensus sequence TGTCTC. This sequence, known as the

Auxin-Responsive Element, or *AuxRE*, has been found capable of conferring auxin-regulated gene expression to reporter constructs (Ulmasov *et al.*, 1995). The identification of the *AuxRE* sequence led to the isolation of the *Arabidopsis* *ARF1* gene in a yeast one-hybrid screen (Ulmasov *et al.*, 1997a). Subsequent genetic, genomic, and molecular studies have led to the identification of 23 *ARF* genes in *Arabidopsis* (Liscum & Reed, 2002).

ARF-mediated transcriptional regulation has been extensively studied in protoplast co-transfection assays employing reporter constructs. ARF proteins share a conserved N-terminal DNA-binding domain that mediates binding to the *AuxRE* sequence. When fused to the VP16 transcriptional activation domain, this N-terminal domain is sufficient to target the ARF fusion protein to an *AuxRE*-containing promoter (Tiwari *et al.*, 2003). Auxin does not appear to influence DNA binding. The middle regions (MR) of ARF proteins are highly divergent, with some ARFs possessing serine-rich (S-rich) and others glutamine-rich (Q-rich) MRs. In all cases examined to date, the S-rich ARFs repress auxin-responsive gene expression in protoplast transfection assays, whereas the Q-rich ARFs act as transcriptional activators (Tiwari *et al.*, 2003). This functional divergence of the ARF transcription factors can be attributed to the disparate MRs since these domains are sufficient to either activate or repress transcription when targeted to a heterologous promoter as a GAL4 DNA-binding domain fusion protein (Tiwari *et al.*, 2003).

The majority of ARF proteins also contain a C-terminal dimerization domain highly related to motifs III and IV of Aux/IAA-proteins (Fig. 2.2). These C-terminal domains mediate homo- and heterodimer formation between ARF proteins as well as heterodimerization with Aux/IAA-proteins (Kim *et al.*, 1997; Ulmasov *et al.*, 1997b). While *in vitro* DNA-binding assays with a palindromic *AuxRE* repeat suggested that ARF-ARF dimerization might facilitate the binding of these transcription factors to their target site (Ulmasov *et al.*, 1999), more recent protoplast transfection assays employing ARF derivatives lacking the C-terminal dimerization elements clearly indicate that ARF dimerization is not essential for *AuxRE* binding (Tiwari *et al.*, 2003).



**Figure 2.2** Conserved domains of Aux/IAA and ARF proteins. Domains III and IV are found in both Aux/IAA and ARF proteins and mediate homo- and heterodimer formation. In *Arabidopsis*, ARF5, -6, -7, -8, and -19 contain Q-rich MRs while the remaining ARFs possess S-rich MRs. S-rich ARFs can be further classified by whether their MR is enriched for only Ser, Ser and Leu, Ser and Gly, or Ser and Pro. *ARF23* has an internal stop codon and is likely a pseudogene.

In the case of the activating ARFs, dimerization with Aux/IAA–proteins provides hormonal control of ARF transcriptional activity. Several members of the Aux/IAA family can interact with Q-rich ARFs and repress transcriptional activation in protoplast assays (Tiwari *et al.*, 2003). Aux/IAA domain I, recently shown to be capable of acting as a transcriptional repressor, may be responsible for this negative regulation although the mechanism remains to be elucidated (Tiwari *et al.*, 2004). In response to an auxin stimulus, Aux/IAA–proteins are degraded by the 26S proteasome (Gray *et al.*, 2001). This reduction in Aux/IAA–protein levels relieves the repression on the Q-rich ARFs, resulting in increased transcription of auxin-responsive genes. Consistent with such a model, dominant gain-of-function mutations have been isolated in several *Aux/IAA* genes that confer dramatic auxin-related phenotypes and reduced auxin response (Table 2.1). Molecular studies have found that all of these mutations map to the domain II degron sequence and result in a dramatic increase in the stability of the mutant Aux/IAA repressor (Gray *et al.*, 2001; Ramos *et al.*, 2001). In contrast, the loss-of-function mutations in *Aux/IAA* genes that have been described to date confer only subtle or no apparent phenotypes, suggesting that there is considerable functional redundancy among the *Aux/IAA* family members.

Analysis of several ARF mutants of *Arabidopsis* has provided insight into the various auxin-dependent processes these transcription factors regulate. Mutations in *ETTIN/ARF3*, an atypical S-rich ARF that lacks the C-terminal dimerization motifs, confer gynoceium patterning defects similar to those observed in flowers treated with

**Table 2.1** Dominant gain-of-function *Aux/IAA* mutants

Mutant	Domain II lesion <sup>1</sup>	Phenotypes <sup>2</sup>	Reference
<i>axr2/iaa7</i>	VVGWPPVR	Auxin, ABA, and ethylene resistant, dwarf, gravitropism defects	Wilson <i>et al.</i> (1990)
<i>axr3/iaa17</i>	VVGWPPVR	Auxin resistant, increased apical dominance & adventitious rooting, reduced root hairs and gravitropism	Leyser <i>et al.</i> (1996)
<i>axr5/iaa1</i>	IVGWPPVR	Auxin resistant, reduced photo- and gravitropism, no apical hook	Yang <i>et al.</i> (2004)
<i>bdl/iaa12</i>	VVGWPPIG	Seedling lethal, fails to establish embryonic root meristem	Hamann <i>et al.</i> (2002)
<i>iaa28</i>	VVGWPPVR	Dwarf, auxin resistant, reduced lateral root development, bushy	Rogg <i>et al.</i> (2001)
<i>msg2/iaa19</i>	VVGWPPVC	Reduced hypocotyl photo- and gravitropism and lateral root development	Tatematsu <i>et al.</i> (2004)
<i>shy2/iaa3</i>	IVGWPPVR	Short hypocotyl, auxin resistant, long root hairs, reduced lateral roots	Tian and Reed (1999)
<i>slr/iaa14</i>	VVGWPPVR	No lateral roots, auxin resistant, gravitropism defects, few root hairs	Fukaki <i>et al.</i> (2002)

<sup>1</sup>The amino acids in the domain II core affected in the various gain-of-function Aux/IAA alleles are indicated in bold.

<sup>2</sup>Only a partial list of phenotypes is presented.

the auxin transport inhibitor NPA (Nemhauser *et al.*, 2000). A screen for mutations restoring ethylene-mediated apical hook formation to the *hookless 1* (*hls1*) mutant identified *HOOKLESS SUPPRESSOR 1* (*HSS1/ARF2*) (Li *et al.*, 2004). Ethylene and light act through *HLS1* to regulate protein levels of this S-rich ARF to mediate differential cell elongation in the hypocotyl. While *arf2* mutants exhibit several phenotypes including increased size of several organs, agravitropic stems, and delayed senescence and abscission, microarray analysis using RNA prepared from 7-day-old *arf2* seedlings did not detect any difference from wild-type in auxin-regulated gene expression (Okushima *et al.*, 2005a). T-DNA insertion mutants in most of the remaining S-rich ARFs have recently been characterized and do not confer any obvious phenotypes suggesting functional redundancy (Okushima *et al.*, 2005b). The recent demonstration that *arf10 arf16* double mutants, but neither single mutant, exhibit agravitropic root growth due to aberrant root cap cell differentiation supports this possibility (Wang *et al.*, 2005).

In the case of the Q-rich activating ARFs, mutations in *MONOPTEROUS* (*MP/ARF5*) confer defects in embryo patterning with severe alleles lacking all basal structures (Hardtke & Berleth, 1998). In contrast, mutations in *NON-PHOTOTROPIC HYPOCOTYL 4* (*NPH4/ARF7*) result in diminished tropic responses in the shoot due to reduced auxin-mediated cell expansion (Harper *et al.*, 2000). While the *mp* and *nph4* mutant phenotypes indicate that these two ARFs regulate distinct auxin responses, the recent finding that *nph4* mutations enhance the rootless phenotype of weak *mp* alleles suggests some functional overlap between these two transcriptional activators (Hardtke *et al.*, 2004). ARF19 also appears to act redundantly with ARF7. While *arf19* mutants exhibit no apparent phenotype, *arf19* enhances *nph4* tropism defects, and the double mutants exhibit additional auxin-related phenotypes including auxin-resistant root elongation and reductions in leaf expansion and lateral root development (Okushima *et al.*, 2005b; Weijers *et al.*, 2005; Wilmoth *et al.*, 2005).

The *arf8* mutants exhibit increased apical dominance, lateral root proliferation, and longer hypocotyls in the light (Tian *et al.*, 2004). Such hyper-auxin phenotypes are characteristic of IAA overproduction mutants such as *yucca* and *sur2*, and at first glance appear to contradict the notion that ARF8 is a transcriptional activator of auxin-responsive genes. However, further analysis revealed that the expression of several *GH3* genes encoding IAA conjugating enzymes is reduced in *arf8* mutants, while elevated in plants overexpressing ARF8 (Tian *et al.*, 2004). These data suggest that ARF8 may specifically be involved in a negative-feedback pathway controlling IAA levels via activation of GH3 expression and suggest a close connection between auxin signaling and auxin homeostasis.

Although the analysis of *ARF* and *Aux/IAA* mutants suggests considerable functional redundancy among the individual members of the respective gene families, the fact that several *arf* mutants exhibit specific auxin-related defects clearly indicates some functional specialization. Likewise, the phenotypes of the different dominant gain-of-function *Aux/IAA* mutants that have been characterized vary considerably. All of the ARF proteins thus far tested bind to a common regulatory sequence, the *AuxRE*. Additionally, at least among the activating ARFs, *Aux/IAA*-proteins do not appear



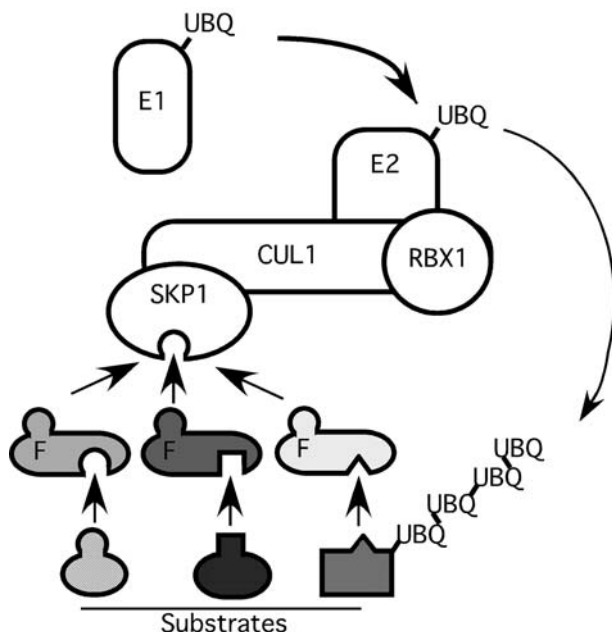
to exhibit a high degree of interaction specificity. For example, several different Aux/IAA–proteins were found to equivalently repress ARF5- and ARF7-mediated transcriptional activation of a reporter construct when co-expressed in carrot protoplasts (Hardtke *et al.*, 2004). This begs the question of how specific auxin responses are generated.

Recent work suggests that this specificity may be achieved in large part by the expression patterns of pairs of Aux/IAA and ARF proteins. Dominant gain-of-function mutations in the *BODENLOS* (*BDL/IAA12*) Aux/IAA gene result in embryo development defects virtually identical to loss-of-function mutations in *MP/ARF5*, suggesting that BDL regulates MP activity (Hamann *et al.*, 2002). In contrast, equivalent domain II mutations in *SHY2/IAA3* confer a short hypocotyl phenotype, but embryogenesis is unaffected suggesting it regulates a distinct ARF. Weijers *et al.* (2005) employed a promoter-swap strategy to determine whether differential transcriptional regulation of these two Aux/IAA genes contributed to the distinct mutant phenotypes. Remarkably, transgenic seedlings expressing the dominant *shy2-2* allele from the *BDL* promoter exhibited an embryo phenotype qualitatively identical to *bdl* mutants. Reciprocally, hypocotyl elongation and shoot development in *pSHY2::bdl* plants were strikingly similar to *shy2-2* mutants. These findings suggest that the SHY2 and BDL proteins are functionally equivalent for these specific auxin responses, and that transcriptional control of Aux/IAA gene expression is a major determinant for directing which auxin responses are regulated by a specific Aux/IAA family member. However, it should also be noted that the same study found strong evidence for functional specialization of Aux/IAA–proteins. Whereas the *pSHY2::bdl* transgene was able to phenocopy the shoot phenotypes of *shy2-2* plants, the auxin-resistant and agravitropic root growth phenotypes characteristic of *shy2-2* seedlings were not observed in the transgenics.

Although additional study is needed, the regulated expression of specific Aux/IAA and ARF family members undoubtedly plays a large role in determining developmental specificity. Several recent studies suggest that post-transcriptional control of ARF expression is also involved in defining expression patterns. Both micro-RNAs (miRNAs) and trans-acting short-interfering RNAs (tasi-RNAs) have recently been identified that direct the cleavage of several ARF transcripts (Mallory *et al.*, 2005; Wang *et al.*, 2005; Williams *et al.*, 2005). For example, the miR160 miRNA targets *ARF10*, *ARF16*, and *ARF17* transcripts. Constitutive overexpression of miR160 results in root cap defects similar to those observed in *arf10 arf16* double mutants, while expression of cleavage-resistant forms of ARF16 or ARF17 confers numerous auxin-related defects, demonstrating that miRNAs likely play a crucial role in restricting ARF expression domains (Mallory *et al.*, 2005; Wang *et al.*, 2005).

### 2.3.3 Regulation of auxin response by the SCF<sup>TIR1</sup> ubiquitin–ligase

The genetic identification and analysis of auxin-resistant mutants in *Arabidopsis* has proven invaluable in elucidating the molecular mechanisms underlying auxin action. Molecular and biochemical studies of the gene products affected by these mutations have positioned the SCF<sup>TIR1</sup> ubiquitin–ligase complex as a central regulator of auxin



**Figure 2.3** The SCF ubiquitin–ligase model. F-box proteins (F) bind to the core SCF subunits (SKP1, CUL1, and RBX1) and recruit specific substrates to the complex, facilitating ubiquitinylation by the associated E2 enzyme. Ubiquitin conjugation targets the substrates for proteolysis by the 26S proteasome. E1 = ubiquitin-activating enzyme; E2 = ubiquitin-conjugating enzyme.

signaling. Ubiquitin–ligases, or E3 enzymes, catalyze the conjugation of ubiquitin to substrate proteins. Once ubiquitinylated, these substrates are targeted for proteolysis by the 26S proteasome.

SCF-type ubiquitin–ligases comprise the largest family of E3 enzymes in *Arabidopsis* (Gagne *et al.*, 2002). These multi-subunit enzymes contain *Skp1*, *Cullin1*, an *F*-box protein, and the small RING domain protein *Rbx1/Roc1/Hrt1* (Deshaies, 1999). *CUL1* acts as a scaffold protein, binding *RBX1* through its C-terminal domain and *SKP1* via its N-terminal domain. *F*-box proteins bind to this hetero-trimeric core via an interaction between their *F*-box domain and the *SKP1* protein, and function as interchangeable adaptor subunits that recruit specific substrates to the SCF complex for ubiquitinylation (Fig. 2.3).

*TIR1* is one of approximately 700 *F*-box proteins encoded in the *Arabidopsis* genome (Gagne *et al.*, 2002). Mutations in *TIR1* confer modest auxin-response defects including auxin-resistant root growth and reduced lateral root development (Ruegger *et al.*, 1998), and molecular studies confirmed *TIR1* as a *bona fide* *F*-box protein by demonstrating that it assembles into a complex with *Arabidopsis* orthologs of the core SCF subunits (Gray *et al.*, 1999, 2002). Mutations in the *AXR6* (*AUXIN RESISTANT 6*) gene encoding the *CUL1* subunit have also been isolated in auxin-resistance screens (Hellmann *et al.*, 2003; Quint *et al.*, 2005), and reverse genetic approaches have been



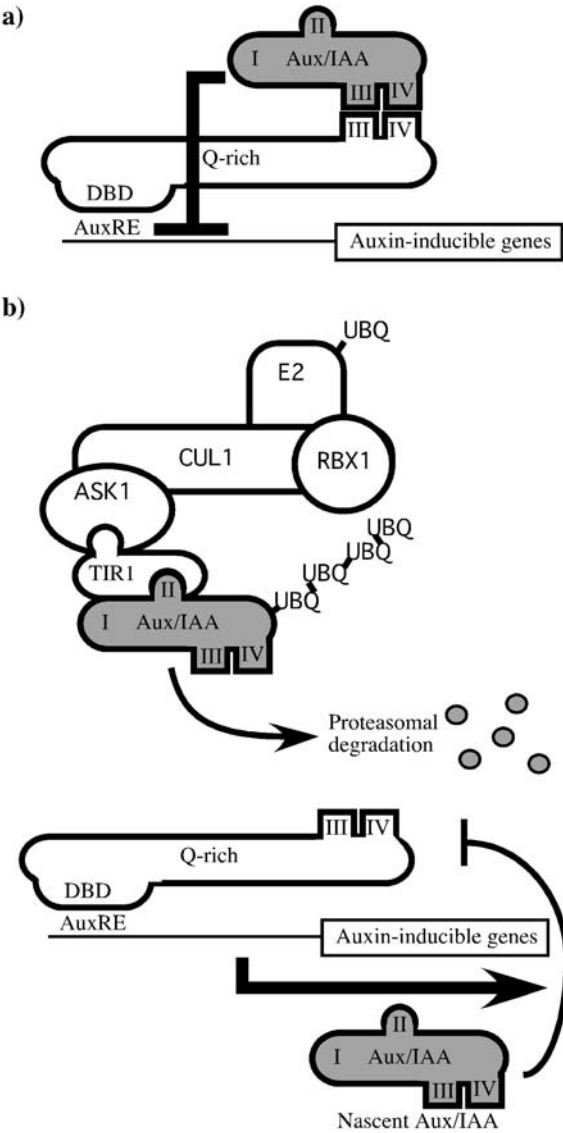
used to demonstrate that the *ASK1* (*Arabidopsis* *SKP1*-like 1) and *RBX1* genes are also required for auxin response (Gray *et al.*, 1999, 2002). Unlike mutations in *TIR1*, mutations or transgenes perturbing core SCF subunits do not specifically affect auxin signaling but instead confer pleiotropic phenotypes, consistent with their participation in additional SCF complexes. These findings established the SCF<sup>TIR1</sup> ubiquitin–ligase complex as a positive regulator of auxin response and suggested a model involving the SCF<sup>TIR1</sup>-mediated ubiquitinylation of a repressor of auxin signaling.

The genetic isolation of several dominant gain-of-function alleles of *Aux/IAA* genes in auxin-resistance screens first implicated the *Aux/IAA*–proteins as repressors of auxin response. As detailed in Table 2.1, molecular analysis revealed that all of these dominant lesions affected amino acids in the highly conserved core of domain II. Early studies on *Aux/IAA*–proteins indicated that these proteins were highly unstable (Abel *et al.*, 1994). This instability is due to domain II, which acts as a degron, targeting *Aux/IAA*–proteins for ubiquitinylation by the SCF<sup>TIR1</sup> complex in response to an auxin stimulus. This was elegantly demonstrated using a combination of molecular, genetic, and biochemical approaches (Gray *et al.*, 2001; Ramos *et al.*, 2001; Zenser *et al.*, 2001). First, domain II confers auxin-regulated instability to luciferase and  $\beta$ -glucuronidase fusion proteins *in vivo*. Second, TIR1 binds to *Aux/IAA*–proteins in cell-free extracts in an auxin-regulated fashion. This binding requires domain II and is disrupted by the gain-of-function *Aux/IAA* mutations. And third, mutations affecting the SCF<sup>TIR1</sup> complex stabilize endogenous *Aux/IAA*–proteins, as well as the domain II–fusion reporter proteins *in vivo*.

These and other findings suggest that in response to an auxin stimulus, *Aux/IAA*–proteins are recruited to the SCF<sup>TIR1</sup> complex and ubiquitinylated. Subsequent *Aux/IAA* degradation by the 26S proteasome derepresses the ARF transcription factors, thus enabling auxin-dependent transcriptional changes (Fig. 2.4). Since domain II mutant derivatives are not recognized by TIR1, these stabilized repressors are immune to this auxin-regulated proteolysis and constitutively repress of ARF activity. While it may seem counterintuitive that auxin promotes both the proteolysis of *Aux/IAA*–proteins and increased *Aux/IAA* gene transcription, this latter response is likely a form of negative feedback that attenuates the auxin signal, ensuring a transient auxin response.

### 2.3.4 Regulation of SCF<sup>TIR1</sup> activity

The analysis of additional auxin-resistant mutants has identified several regulatory components modulating SCF<sup>TIR1</sup> function. *axr1* mutants exhibit severe auxin response defects (Lincoln *et al.*, 1990). *AXR1* encodes a protein related to the amino-terminal half of the ubiquitin-activating enzyme (Leyser *et al.*, 1993), and biochemical studies have revealed AXR1 interacts with the E1-LIKE CONJUGATING ENZYME RELATED 1 (ECR1) protein. Together, AXR1 and ECR1 form a RELATED TO UBIQUITIN (RUB) activating enzyme. *Arabidopsis* encodes three RUB proteins (known as NEDD8 in mammals) sharing approximately 60% sequence identity with ubiquitin. Once activated, RUB is transferred to the RUB1 CONJUGATING ENZYME 1 (RCE1), which using RBX1 as a RUB E3 ligase, catalyzes the addition of



**Figure 2.4** Regulation of auxin response by the *SCF<sup>TIR1</sup>* ubiquitin-ligase. (a) Under sub-threshold auxin concentrations, Aux/IAA-proteins are stable and dimerize with ARF transcriptional activators, repressing the activation of auxin-inducible genes. (b) Upon an auxin stimulus, the TIR1 F-box protein recruits the Aux/IAA repressors to the *SCF<sup>TIR1</sup>* complex, which ubiquitinylates the Aux/IAA-proteins, thus targeting them for degradation by the 26S proteasome. The resulting decline in Aux/IAA levels derepresses the Q-rich ARFs, freeing them to activate auxin-inducible genes. In addition to genes involved in specific auxin responses (i.e. lateral root development), the *Aux/IAA* genes are also induced. Synthesis of nascent Aux/IAA-proteins may constitute a negative feedback loop that ensures a transient response by restoring repression on the ARF proteins.

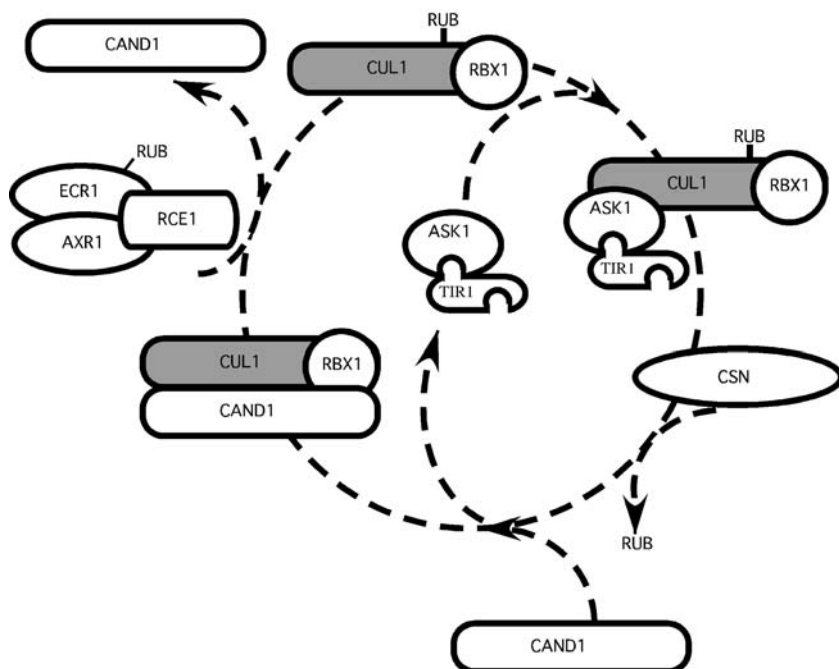
RUB to a lysine residue near the C-terminus of the CUL1 subunit of the SCF<sup>TIR1</sup> complex (Del Pozo & Estelle, 1999; Gray *et al.*, 2002; Dharmasiri, S. *et al.*, 2003). Unlike ubiquitin, RUB conjugation does not result in substrate proteolysis, but rather is a regulatory modification required for SCF ubiquitin–ligase activity as demonstrated by the finding Aux/IAA stability is increased in RUB pathway mutants (Gray *et al.*, 2001).

The RUB modification of CUL1 is a dynamic process. The SCF interacts with the COP9 signalosome (CSN), an 8-subunit complex resembling the 26S proteasome lid (Schwechheimer *et al.*, 2001). The CSN possesses an isopeptidase activity that cleaves the RUB modifier off of CUL1. Surprisingly, plants with impaired CSN function exhibit diminished auxin response and increased Aux/IAA stability indicating that like RUB conjugation, RUB cleavage off of CUL1 is also required for optimal SCF<sup>TIR1</sup> activity (Schwechheimer *et al.*, 2001). Also consistent with the notion that RUB cleavage plays an important role in SCF function, plants overexpressing RBX1 exhibit hyper-modification of CUL1, yet display several auxin response defects and reduced SCF<sup>TIR1</sup> activity (Gray *et al.*, 2002).

The molecular function of RUB modification is unclear. Biochemical assays suggest RUB modification of CUL1 may enhance SCF activity *in vitro* by facilitating interactions with ubiquitin-charged E2 enzymes. An additional possibility has emerged with the identification of CULLIN ASSOCIATED AND NEDD8 DISSOCIATED 1 (CAND1). Biochemical studies with human CUL1 identified CAND1 as a CUL1-binding protein. CAND1 specifically interacts with unmodified cullin, and can be dissociated by the RUB modification of CUL1 *in vitro*. Furthermore, CAND1 and SKP1 binding to CUL1 are mutually exclusive, suggesting a model whereby CAND1 sequesters a fraction of the CUL1 pool, thus negatively regulating ubiquitin–ligase activity by preventing SCF complex assembly. Genetic studies of *Arabidopsis cand1* mutants, however suggest a more complicated scenario. Rather than exhibiting increased SCF<sup>TIR1</sup> activity as might be expected, *cand1* mutants are defective in Aux/IAA proteolysis and display diminished auxin response phenotypes similar to SCF mutants (Cheng *et al.*, 2004; Chuang *et al.*, 2004; Feng *et al.*, 2004). To reconcile these observations, it has been suggested that the RUB pathway, CAND1, and the CSN sustain SCF activity *in vivo* by promoting cycles of SCF assembly and disassembly (Fig. 2.5) (Petroski & Deshaies, 2005). Although such a cycle is not required for SCF activity in *in vitro* ubiquitinylation assays, it may be essential in a cellular context where many F-box proteins are competing for access to the common core SCF subunits. While additional studies are required to test this model, it is clear that CAND1, the CSN, and the RUB pathway are intimately linked and required for normal SCF<sup>TIR1</sup> function. SGT1b, which also interacts with SCF complexes and is required for SCF<sup>TIR1</sup> activity *in vivo* has also been suggested to potentially regulate SCF assembly (Gray *et al.*, 2003).

### 2.3.5 Identification of an auxin receptor

Central to understanding the molecular basis of auxin action is the elucidation of how auxin is perceived. Classical studies provide support for both plasma membrane and



**Figure 2.5** A model for  $SCF^{TIR1}$  regulation by the cyclic RUB modification of CUL1. The active  $SCF^{TIR1}$  complex containing RUB-modified CUL1 is shown at the right. The CSN complex can cleave the RUB modifier from CUL1, thus facilitating CAND1 binding to CUL1 and SCF disassembly. Conjugation of RUB to CUL1 by the AXR1–ECR1 and RCE1 enzymes frees CUL1 from CAND1, promoting re-assembly of the active complex. Genetic and biochemical studies have shown that all of the components depicted in this figure are required for optimal  $SCF^{TIR1}$  activity *in vivo*.

intracellular sites of auxin perception. Biochemical efforts to isolate the auxin receptor identified AUXIN BINDING PROTEIN 1 (ABP1) as a protein that binds biologically active auxins with high affinity (reviewed in Napier *et al.*, 2002). Originally purified from maize coleoptiles, this 22 kDa protein has subsequently been found in many species and is apparently unique to the plant kingdom. The protein sequence of ABP1 reveals little functional information. Consistent with the presence of a C-terminal KDEL endoplasmic retention motif, the majority of ABP1 is ER localized. However, a small fraction escapes to the cell surface, and several lines of evidence implicate plasma membrane associated ABP1 in early auxin-mediated electrophysiologic responses (Napier *et al.*, 2002). Genetic approaches to elucidate ABP1 function have found that its overexpression promotes auxin-mediated cell expansion (Jones *et al.*, 1998). Furthermore, *Arabidopsis abp1* null mutants display an early embryo-lethal phenotype, indicating that ABP1 is an essential gene (Chen *et al.*, 2001). However, the molecular activity of ABP1 remains undetermined, and it does not appear to be involved in the  $SCF^{TIR1}$ -mediated signaling pathway (Dharmasiri, N. *et al.*, 2003).

The demonstration that auxin, when added to a crude protein lysate, could promote the binding of Aux/IAA-proteins to the SCF<sup>TIR1</sup> complex indicated that an auxin receptor was functional in these extracts and provided a new approach for receptor identification. Studies of several F-box protein-substrate interactions in yeast and animal systems have revealed that in virtually every case, the substrate must be post-translationally modified before it can be recognized by its cognate F-box protein. Generally, this involves substrate phosphorylation by a stimulus-activated kinase, although other types of modifications have also been reported (Petroski & Deshaies, 2005). Since domain II is not only necessary, but is sufficient for auxin-induced binding to TIR1, it was hypothesized that an auxin-activated kinase or other enzyme modified domain II, thus targeting the Aux/IAA-protein for SCF-mediated ubiquitinylation and ultimately degradation. However, the domain II degron does not contain any essential phosphorylatable residues, and no detectable modifications were detected by MS analysis following incubation of the domain II peptide with auxin-treated extracts (Ramos *et al.*, 2001; Kepinski & Leyser, 2004). This led to the alternative possibility that auxin acted through TIR1.

Two recent reports have implicated the TIR1 F-box protein as an auxin receptor when it was found that radiolabeled IAA added to crude *Arabidopsis* extracts co-purifies with the SCF<sup>TIR1</sup>-Aux/IAA complex (Dharmasiri *et al.*, 2005a; Kepinski & Leyser, 2005). This binding is both saturable and specific for active auxins, with an estimated  $K_d$  in the 20–80 nM range. While this alone only suggests that an auxin receptor is associated with SCF<sup>TIR1</sup>, the finding that TIR1 expressed in animal cells also interacts with a recombinant Aux/IAA-protein in an auxin-dependent manner is more telling. As TIR1 and the Aux/IAA-protein are the only plant-derived components in these assays, it seemed that one of these proteins must be responsible for auxin binding. Furthermore, since a 17-amino-acid domain II peptide can substitute for the intact Aux/IAA-protein, and an auxin pretreatment of TIR1, but not of the Aux/IAA-protein, also promotes the TIR1-Aux/IAA interaction *in vitro* (Kepinski & Leyser, 2004; 2005), the TIR1 F-box protein appears to be the long-sought auxin receptor. It should be noted however, that direct IAA binding to purified TIR1 in the absence of an Aux/IAA-protein remains to be demonstrated.

The fact that *tir1* null mutants exhibit a relatively weak auxin response defect compared to *axr1* or dominant Aux/IAA mutants suggested that TIR1 might not be the only auxin receptor. Indeed, three additional AUXIN SIGNALING F-BOX proteins, AFB1, AFB2, and AFB3, displaying 60–70% sequence identity to TIR1 were recently shown to exhibit auxin-dependent binding to Aux/IAA-proteins *in vitro* (Dharmasiri *et al.*, 2005b). Correspondingly, the introduction of *afb* mutations into *tir1* plants results in a progressive reduction in auxin response, culminating with *tir1 afb1 afb2 afb3* quadruple mutants exhibiting a seedling-lethal phenotype similar to *mp* and *bdl* mutants. Aux/IAA pull-down assays with quadruple mutant extracts detected no saturable IAA-binding activity, indicating that auxin binding is dependent on these F-box proteins (Dharmasiri *et al.*, 2005a). The molecular details of auxin binding to the TIR1/AFB receptors remain to be elucidated, including the identification of the binding site and how hormone binding facilitates interactions with the Aux/IAA-proteins.

## 2.4 Conclusions and future perspectives

The identification of the TIR1/AFB family of receptors fills in a crucial piece of the auxin puzzle. Though many details remain to be worked out, we now have a framework for a potentially complete signaling pathway leading from hormone perception to transcriptional response. Can such a simple pathway – with the TIR1/AFB receptors sensing IAA to trigger the ubiquitinylation of Aux/IAA–proteins and thus derepressing the ARF transcription factors – account for the multitude of responses elicited by the hormone? Differential expression of the many *ARF* and *Aux/IAA* genes coupled with the large number of potential combinatorial interactions between these proteins could potentially provide both developmental specificity and the complexity needed for a large array of auxin responses. However, it is also likely that additional auxin signaling pathways await discovery, as some responses appear too quickly to result from transcriptional changes (Hager, 2003). Analysis of auxin response in plants lacking the *TIR1* and *AFB* genes may be a useful approach for identifying such pathways. Furthermore, the SAUR proteins, ABP1, a MAP kinase (Mockaitis & Howell, 2000), the IBR5 dual specificity phosphatase (Monroe-Augustus *et al.*, 2003), Rac GTPases (Tao *et al.*, 2002), and a host of other potential signaling factors have been implicated in auxin signaling but are presently without a home in current models for auxin response, clearly indicating that there is much yet to learn about the mechanics of auxin signaling.

Likewise, Aux/IAA and ARF function are only beginning to be understood. The number of potential Aux/IAA–Aux/IAA, Aux/IAA–ARF, and ARF–ARF dimer combinations is massive, yet so far the only interaction ascribed any biologic significance is the negative regulation of the activating ARFs by Aux/IAA–proteins. Are all Aux/IAAs negative regulators? Do Aux/IAA–proteins regulate the S-rich ARFs? If not, are these repressing ARFs subject to hormonal regulation, and if so, how? The answers to these questions, as well as the identification of the downstream effector genes that are the targets of ARF regulation will greatly improve our understanding of auxin-regulated growth and development.

Molecular and biochemical processes involved in auxin biology have in common an impressive complexity and, in the case of auxin metabolism, functional redundancy. Key areas of future research will be the understanding of the entire auxin network – IAA biosynthesis and degradation, conjugation, transport, signaling, and response together. Many links between these individual pathways have already been established, such as auxin induction of *GH3* expression and its resultant predicted effects on conjugation and oxidation, however it is likely that many more connections await identification. An even greater challenge is elucidating the coordination of auxin with other signaling pathways. IAA does not act in isolation, but in concert with other phytohormones and developmental and environmental signals to control plant growth and development. Understanding the integration of auxin with these other pathways is a daunting task, but is necessary to truly understand the role of this amazing hormone in plant biology.

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## 3 Integration of brassinosteroid biosynthesis and signaling

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### 3.1 Introduction

In less than a decade, our understanding of plant steroid hormone biosynthesis and signaling has advanced to the point that this class of hormones can be argued to have one of the best characterised biosynthesis and signaling pathways. Brassinolide (BL) is the most bioactive form of these hormones, which are generically referred to as brassinosteroids (BRs). Genetic approaches have identified dwarf mutants that are defective in BR synthesis and signaling. The dwarf phenotype, as shown in Fig. 3.1, highlights the essential role of BRs in plant development, which involves the regulation of growth, photomorphogenesis, fertility and stress resistance (for reviews see: Clouse & Sasse, 1998; Altmann, 1999). BR regulation of these physiological functions indicates the importance of maintaining optimal levels of BRs via the coordination of biosynthesis and signaling.

Many recent reviews have discussed BR biosynthesis, metabolism and signaling in model systems (Clouse, 2002; Fujioka & Yokota, 2003; Li, 2005; Li & Deng, 2005; Vert *et al.*, 2005) and crops (Bishop, 2003). Here we aim to provide an account of BR synthesis and signaling, developing a theme of how biosynthesis is regulated by BR signaling. A key concept in this regulation is that homeostatic levels of the hormone are maintained by BR-dependent transcriptional control of the genes involved in BR metabolism (Fig. 3.2). This control mechanism lowers the hormone content when BR levels are too high by reducing the transcription of biosynthetic genes and/or increasing the transcription of catabolic genes.

This review will therefore include accounts of BR biosynthesis and metabolism through to the signaling processes that occur in the presence or absence of BRs. The focus will be on discussing the progress made in the model plant *Arabidopsis thaliana*. Where appropriate, key advances achieved in other species will also be included.

### 3.2 Metabolism

#### 3.2.1 Biosynthesis

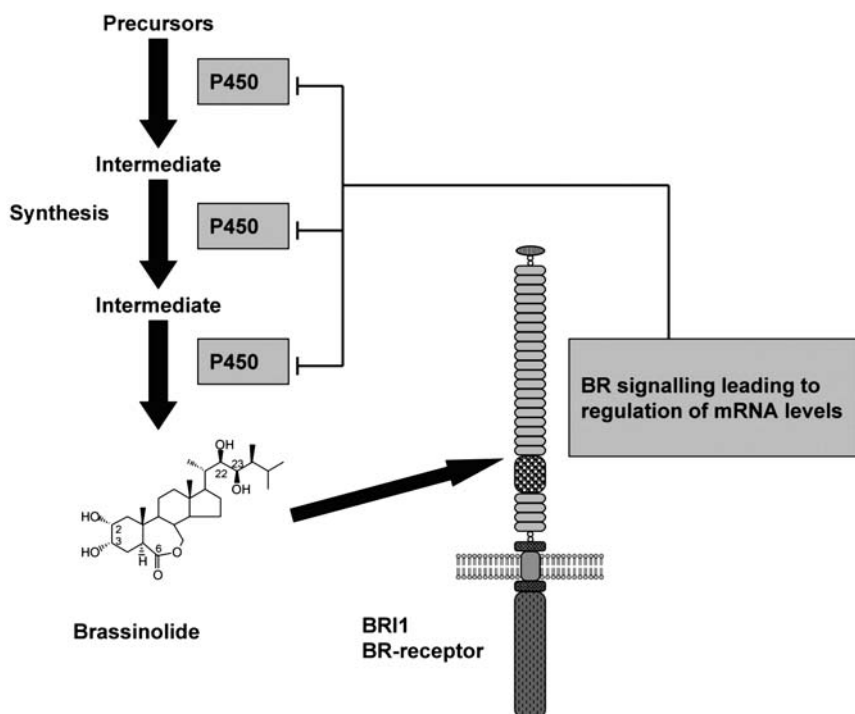
BRs are synthesised from C<sub>27</sub>, C<sub>28</sub> and C<sub>29</sub> phytosterol precursors that differ from each other by their aliphatic substituents at the C-24 position. Campesterol, a C<sub>28</sub> sterol is the main precursor for BR synthesis. Phytosterol synthesis, including that



**Figure 3.1** *BR* deficiency results in severe dwarf phenotype. The *BR*-biosynthetic *cpd* mutant of *Arabidopsis* (right) is shown next to a wild-type Col-0 plant (left). Scale bar represents 5 cm.

of campesterol, has been reviewed in detail by Fujioka and Yokota (2003), therefore the focus of this section will be on the reactions downstream of campesterol. The biosynthetic pathway of BRs has been deduced by the analysis of conversion products obtained from radiolabelled metabolites fed to cell suspension cultures of *Catharanthus roseus*. These studies revealed that BR synthesis proceeds towards BL via two major routes, namely the early and late C-6 oxidation pathways (Choi *et al.*, 1997). Subsequently, this model of BR biosynthesis (Fig. 3.3) was confirmed in *Catharanthus* (Fujioka *et al.*, 2000) and *Arabidopsis* (Noguchi *et al.*, 2000).



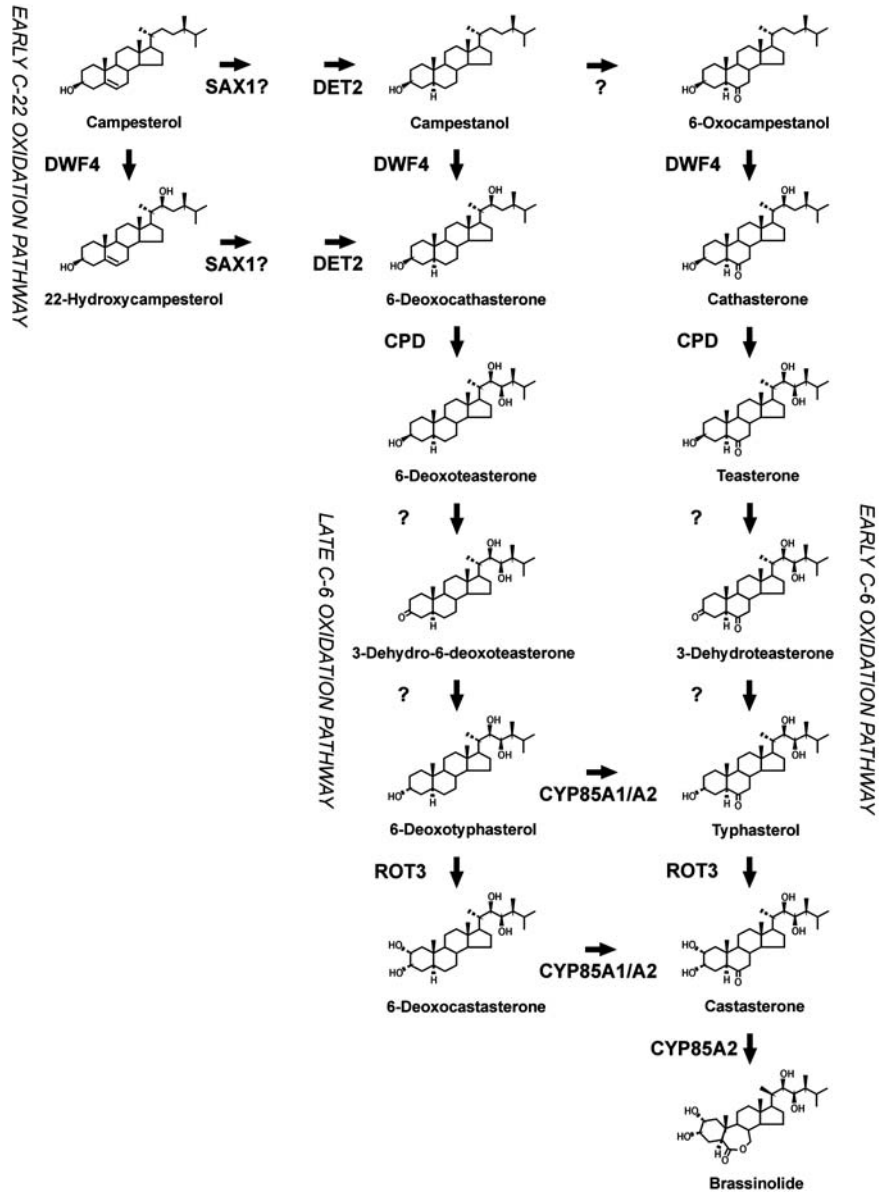


**Figure 3.2** *Transcriptional feedback regulation.* Model showing the feedback mechanism regulating BL synthesis. The BRASSINOSTEROID INSENSITIVE 1 (BR1) leucine rich repeat receptor-like kinase present in the plasma membrane binds BL. A signaling cascade is activated that down-regulates the transcripts of cytochrome P450 genes involved in BL synthesis.

The isolation of BR-deficient mutants and their molecular genetic characterisation has been instrumental in identifying the genes involved in BR biosynthesis and clarifying the enzymatic role of their products. Currently, most of the biosynthetic genes and their functions have been characterised in *Arabidopsis*, and some of their orthologues have been described from other species (Table 3.1). The following provides an account of these enzymes. However, the enzymes responsible for early C-6 oxidation and the isomerisation of the C-3 hydroxyl group are yet to be identified.

### 3.2.1.1 *DET2*

*DEETIOLATED 2 (DET2)* was one of the first BR-biosynthetic genes to be identified in *Arabidopsis*. Its lesion results in a typical BR-deficient phenotype, characterised by severe dwarfism, constitutive photomorphogenesis, delayed flowering/senescence and reduced male fertility (Chory *et al.*, 1991; Li *et al.*, 1996). *DET2* encodes a steroid 5 $\alpha$ -reductase, representing the only known non-cytochrome P450 enzyme (P450) of BR biosynthesis. The enzymatic role of *DET2* was determined by *in vivo* feeding assays using deuterated substrates, as well as the analysis of BR intermediates in the



**Figure 3.3** The biosynthetic pathways of BRs. The scheme shows the synthesis routes of the functionally most important C<sub>28</sub>-BRs. Conversion steps are designated by arrows, and symbols at the arrows denote the *Arabidopsis* enzymes responsible for each reaction. The early and late C-6 oxidation, as well as the early C-22 oxidation pathways are indicated.

**Table 3.1** Genes of BR metabolism

Genes (alleles)	Function	Species	References
BR biosynthesis			
<i>DET2</i>	5 $\alpha$ -reductase	<i>Arabidopsis</i>	Li <i>et al.</i> (1996), Noguchi <i>et al.</i> (1999b)
<i>LK</i>	5 $\alpha$ -reductase	Pea	Nomura <i>et al.</i> (2004)
<i>LeDET2</i>	5 $\alpha$ -reductase	Tomato	Rosati <i>et al.</i> (2005)
<i>PnDET2</i>	5 $\alpha$ -reductase	<i>Pharbitis nil</i>	Suzuki <i>et al.</i> (2003)
<i>SAX1</i>	3-oxidase, isomerase	<i>Arabidopsis</i>	Ephritikhine <i>et al.</i> (1999a & b)
<i>DWF4/CYP90B1</i>	22-hydroxylase	<i>Arabidopsis</i>	Choe <i>et al.</i> (1998, 2001)
<i>CPD/CYP90A1 (CBB3, DWF3)</i>	23-hydroxylase	<i>Arabidopsis</i>	Szekeres <i>et al.</i> (1996), Mathur <i>et al.</i> (1998)
<i>COS10/CYP90A2</i>	23-hydroxylase?	Mung bean	Yang, M.-T. <i>et al.</i> (2005)
<i>dpy</i>	23-hydroxylase?	Tomato	Koka <i>et al.</i> (2000)
<i>ROT3/CYP90C1</i>	2-hydroxylase	<i>Arabidopsis</i>	Kim <i>et al.</i> (1998) and Kim, G.-T. <i>et al.</i> (2005)
<i>CYP90D1</i>	3-oxidase, isomerase?	<i>Arabidopsis</i>	Kim, G.-T. <i>et al.</i> (2005a)
<i>D2/CYP90D2</i>	3-oxidase, isomerase?	Rice	Hong <i>et al.</i> (2003)
<i>CYP90D3</i>	3-oxidase, isomerase?	Rice	Hong <i>et al.</i> (2003)
<i>DDWF1/CYP92A6</i>	2-hydroxylase?	Pea	Kang <i>et al.</i> (2001)
<i>CYP85A1</i>	6-oxidase	<i>Arabidopsis</i>	Shimada <i>et al.</i> (2001), Castle <i>et al.</i> (2005), Kwon <i>et al.</i> (2005)
<i>Dwarf/CYP85A1</i>	6-oxidase	Tomato	Bishop <i>et al.</i> (1996), Bishop <i>et al.</i> (1999), Montoya <i>et al.</i> (2005)
<i>BRD1/CYP85A1</i>	6-oxidase	Rice	Hong <i>et al.</i> (2002), Mori <i>et al.</i> (2002)
<i>CYP85A2</i>	6-oxidase, BL synthase	<i>Arabidopsis</i>	Shimada <i>et al.</i> (2003), Nomura <i>et al.</i> (2005), Kim, T.-W. <i>et al.</i> (2005), Kwon <i>et al.</i> (2005)
<i>CYP85A3</i>	6-oxidase, BL synthase	Tomato	Nomura <i>et al.</i> (2005)
<i>CYP724B1</i>	?	Rice	Tanabe <i>et al.</i> (2005)
BR inactivation			
<i>BAS1/CYP734A1</i>	26-hydroxylase	<i>Arabidopsis</i>	Neff <i>et al.</i> (1999), Turk <i>et al.</i> (2003)
<i>CHI2/CYP72C1</i> ( <i>SHK1/SOB7</i> )	Hydroxylase?	<i>Arabidopsis</i>	Nakamura <i>et al.</i> (2005), Takahashi <i>et al.</i> (2005), Turk <i>et al.</i> (2005)
<i>UGT73C5</i>	UDP- glycosyltransferase	<i>Arabidopsis</i>	Poppenberger <i>et al.</i> (2005)
<i>BNST3 and BNST4</i>	Steroid sulphotransferase SOT	<i>Brassica napus</i>	Rouleau <i>et al.</i> (1999), Marsolais <i>et al.</i> (2004)

*det2* mutant. These studies revealed that DET2 catalyses one of the initial reactions in the BR pathway, namely the conversion of (24R)-24-methylcholest-4-en-3-one to (24R)-24-methyl-5- $\alpha$ -cholestan-3-one (Fujioka *et al.*, 1997; Noguchi *et al.*, 1999b). DET2 is an enzyme of relaxed specificity, allowing 5 $\alpha$ -reduction of not only BR precursors with C<sub>28</sub> and C<sub>27</sub> backbones (Fujioka *et al.*, 2002), but also several animal steroid substrates (Li *et al.*, 1997). These results and sequence similarities shared with animal 5 $\alpha$ -reductases show that DET2 is an ancient enzyme of steroid biosynthesis. Genes encoding orthologues of *DET2* have also been identified in some other plant species. These include *LK* in pea (Nomura *et al.*, 2004), *LeDET2* in tomato (Rosati *et al.*, 2005) and *PnDET2* in *Pharbitis nil* (Suzuki *et al.*, 2003).

### 3.2.1.2 *SAX1*

The dwarf phenotype of the ABA- and IAA-hypersensitive *sax1* (*hypersensitive to abscisic acid and auxin 1*) mutant of *Arabidopsis* can be rescued by 24-epiBL, indicating that the mutation causes BR deficiency (Ephritikhine *et al.*, 1999a). On the basis of rescue assays using intermediates of BR synthesis, *SAX1* was tentatively identified as a steroid C-3 oxidase catalysing the conversion of campesterol to ergost-4-ene-3-one, immediately upstream of the DET2 reaction (Ephritikhine *et al.*, 1999b). The *sax1* mutation was mapped within an approximately 3.7 Mb region at the lower arm of chromosome 1 (Ephritikhine *et al.*, 1999a), but so far the gene has not been identified.

### 3.2.1.3 *DWF4*

The *dwarf4* (*dwf4*) mutant of *Arabidopsis* displays a typical BR-deficient phenotype (Azpiroz *et al.*, 1998). *DWF4*, the gene affected by the mutation has been cloned and shown to encode a P450, CYP90B1 (Choe *et al.*, 1998). In feeding assays C-22-hydroxylated BR intermediates rescued the *dwf4* phenotype, indicating that DWF4/CYP90B1 is responsible for C-22 hydroxylation of the steroid side chain (Choe *et al.*, 1998). Combined gas chromatography and mass spectrometry (GC-MS) analyses of BR intermediates revealed that plants accumulate very high amounts of campestanol, a substrate of DWF4, which suggests that C-22 hydroxylation is one of the rate-limiting reactions of BR synthesis (Nomura *et al.*, 2001). The importance of this reaction is supported by the results of Choe *et al.* (2001) showing enhanced growth in transgenic *Arabidopsis* and tobacco plants that overexpress *DWF4* under the control of the cauliflower mosaic virus 35S promoter. In addition to its crucial role in BR synthesis, DWF4 is the prime target of the triazole-type BR-biosynthesis inhibitors brassinazole (Asami *et al.*, 2001) and triadimefon (Asami *et al.*, 2003).

The natural occurrence of 22-hydroxycampesterol and other C-22-hydroxylated precursors of 6-deoxocathasterone reveals that DWF4 also catalyses reactions in the early C-22-hydroxylation pathway (Fujioka *et al.*, 2002). Recent enzymological studies with heterologously expressed DWF4 indicate that campesterol, rather than campestanol, is the preferred substrate of this enzyme (Fujita *et al.*, 2006).

*DWF4* is expressed primarily in the shoot apex and siliques of *Arabidopsis*, and its mRNA level was found to be very low compared to other BR-biosynthetic transcripts

(Shimada *et al.*, 2003). High BR levels down-regulate *DWF4* by a transcriptional feedback mechanism that controls the expression of all BR-biosynthetic P450 genes in *Arabidopsis* (Choe *et al.*, 2001; Bancos *et al.*, 2002). In contrast to other genes of this group, *DWF4* activity retains its responsiveness to the biosynthesis inhibitor brassinazole in a BR-insensitive mutant background (Tanaka *et al.*, 2005).

#### 3.2.1.4 CPD

Like *dwf4*, *constitutive photomorphogenesis and dwarfism (cpd)* is a T-DNA insertion mutant with extreme dwarf stature (Fig. 3.1) and aberrant skotomorphogenesis (Kauschmann *et al.*, 1996; Szekeres *et al.*, 1996). *CPD* encodes a P450 (CYP90A1) that is 43% identical with CYP90B1 (Choe *et al.*, 1998). Phenotypic rescue experiments using BR intermediates suggest that CPD is a BR C-23 hydroxylase (Szekeres *et al.*, 1996). *CPD* is the most highly expressed BR-biosynthetic gene (Shimada *et al.*, 2003), with preferential activity in cotyledons and expanding leaves (Mathur *et al.*, 1998). The high endogenous levels of the CPD substrate 6-deoxocathasterone indicate that this is an important enzyme of BR synthesis (Nomura *et al.*, 2001).

#### 3.2.1.5 ROT3 and CYP90D1

Compared to *det2*, *dwf4* and *cpd*, the *rotundifolia 3 (rot3)* mutants of *Arabidopsis* display much weaker phenotypes, with only slightly reduced elongation (Kim *et al.*, 1998). In contrast, transgenic plants overexpressing the *ROT3* gene develop elongated leaves and petals (Kim *et al.*, 1999). *ROT3/CYP90C1* was anticipated to function in BR biosynthesis on the basis of its sequence similarity with CPD/CYP90A1 and DWF4/CYP90B1, as well as its down-regulated expression by BRs (Bancos *et al.*, 2002; Goda *et al.*, 2002). Sequence data revealed that the *Arabidopsis* genome also encodes CYP90D1, another P450 protein sharing 47% sequence identity with *ROT3*. *CYP90D1* is also transcriptionally down-regulated by BRs (Bancos *et al.*, 2002; Goda *et al.*, 2002), implicating its P450 product in BR biosynthesis, but a null mutation in this gene does not have an obvious phenotypic effect (Kim, G.-T. *et al.*, 2005). By contrast, a *cyp90c1cyp90d1* double mutant defective in both *ROT3* and *CYP90D1* shows the typical BR-deficient dwarf phenotype (Kim, G.-T. *et al.*, 2005), suggesting that the two enzymes may have redundant functions. Despite this expectation, Kim, G.-T. *et al.* (2005) found that overexpression of *CYP90D1* in the *rot3-1* mutant could not rescue the phenotype. Furthermore, the levels of BR intermediates were affected differently by the *rot3* and *cyp90d1* mutations. Based on the endogenous BR levels in these mutants, *ROT3* was proposed to be a C-2 hydroxylase catalysing the conversion of typhasterol to castasterone, whereas *CYP90D1* was thought to mediate a reaction upstream of the C-2 hydroxylation, possibly the oxidation of teasterone and 6-deoxoteasterone to their respective 3-dehydro derivatives (Kim, G.-T. *et al.*, 2005).

The BR-deficient *ebisu dwarf (d2)* mutation of rice is caused by a lesion in the *D2* gene encoding CYP90D2, which is 54% identical with *Arabidopsis* CYP90D1 at the amino acid sequence level (Hong *et al.*, 2003). Based on the rescue of the *d2* phenotype by BR biosynthesis intermediates and the endogenous BR levels detected in the mutant, CYP90D2 has been proposed to catalyse the conversion of 6-deoxoteasterone

to 3-dehydro-6-deoxoteasterone (Hong *et al.*, 2003). Therefore, this enzyme is a likely orthologue of the *Arabidopsis* CYP90D1. Genomic sequence data indicate that rice also contains CYP90D3, a likely isoform of CYP90D2 with yet unidentified function (Hong *et al.*, 2003).

### 3.2.1.6 *CYP85A1 and CYP85A2*

Mutants at the tomato *dwarf* (*d*) locus are BR responsive and this gene was transposon tagged using an engineered Activator transposable element from maize (Bishop *et al.*, 1996). The *DWARF* gene was shown to encode CYP85A1, a P450 closely related to the BR-biosynthetic P450s of the CYP90 family (Bishop *et al.*, 1996). GC-MS analysis of endogenous BRs from *d<sup>x</sup>* mutants (a strong allele at the *d* locus), did not detect castasterone, but an accumulation of its precursor 6-deoxocastasterone (Bishop *et al.*, 1999). Overexpression of the *D* gene resulted in the increase of castasterone content at the expense of the 6-deoxocastasterone pool. These results suggested that CYP85A1 catalyses the late C-6 oxidation reaction converting 6-deoxocastasterone to castasterone. Subsequently, this was confirmed in functional assays in which the yeast-expressed enzyme oxidises 6-deoxocastasterone to castasterone via a C-6-hydroxylated intermediate (Bishop *et al.*, 1999).

In *Arabidopsis* two *CYP85* genes were identified on the basis of their homology to *D*, rather than the characterisation of mutants (Shimada *et al.*, 2001; 2003). Sequence analysis of *CYP85A1* and *CYP85A2* revealed that they encode closely related P450s sharing 82% amino acid sequence identity. When expressed in yeast, both enzymes catalysed C-6 oxidation of 6-deoxocastasterone and, to a lesser extent, also 6-deoxotyphasterol and 6-deoxoteasterone (Shimada *et al.*, 2001; 2003). In addition to its C-6 oxidase activity, CYP85A2 also catalysed the conversion of castasterone to BL (Kim, T.-W. *et al.*, 2005; Nomura *et al.*, 2005). Mutant analyses indicated that these genes have partially overlapping functions, since only double mutants deficient in both *CYP85A1* and *CYP85A2* displayed the characteristic BR dwarf phenotype (Kwon *et al.*, 2005; Nomura *et al.*, 2005).

The recently discovered CYP85A3 of tomato exhibits both BR C-6 oxidase and BL-synthase activity, thereby indicating that it is an orthologue of the *Arabidopsis* CYP85A2 (Nomura *et al.*, 2005). The analysis of CYP85A3 expression indicates that this enzyme is fruit-specific (Nomura *et al.*, 2005), which is in good agreement with the observation that in tomato BL synthesis is restricted to the fruits (Montoya *et al.*, 2005). This suggests an important role for BL in reproductive development.

### 3.2.1.7 *Other biosynthetic functions*

The exact function of some BR biosynthesis related genes are yet to be clarified. These include:

DARK-INDUCED DWF-LIKE PROTEIN 1 (DDWF1) from pea, which was identified as a specific interactor of the Pra2 dark-induced small GTP-binding protein that is expressed only in etiolated epicotyls (Kang *et al.*, 2001). The *DDWF1* gene encodes CYP92A6, a P450 enzyme that has been proposed to be a BR C-2 hydroxylase. The

importance of this enzyme in BR biosynthesis is not clear because some plants (e.g. *Arabidopsis*) lack CYP92-type P450s (The *Arabidopsis* Genome Initiative, 2000).

The *dumpy* (*dpy*) mutant of tomato has reduced BR content and its dwarf phenotype can be rescued by bioactive BRs. On the basis of BR content and intermediate rescue data, DPY is proposed to function as a C-23 hydroxylase (Koka *et al.*, 2000).

BRASSINOSTEROID, LIGHT AND SUGAR 1 (BLS1) of *Arabidopsis* is required for proper BR and light responsiveness. The phenotype of the *bls1* mutant can be rescued by BR treatment, a feature shared with BR-biosynthetic mutants. The mutation has been mapped within a 1.4 Mb region of chromosome 5 (Laxmi *et al.*, 2004).

DWARF 11 (D11) is a rice P450 enzyme (CYP724B1) related to members of the CYP85 and CYP90 families. BR analysis and intermediate rescue of the *d11* mutant suggest that CYP724B1 is involved in BR synthesis upstream of 6-deoxotyphasterol, but the function of this enzyme remains unclear (Tanabe *et al.*, 2005). Based on sequence similarity, it is likely that the closely related CYP724A1 that has been identified in *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000), also participates in BR biosynthesis.

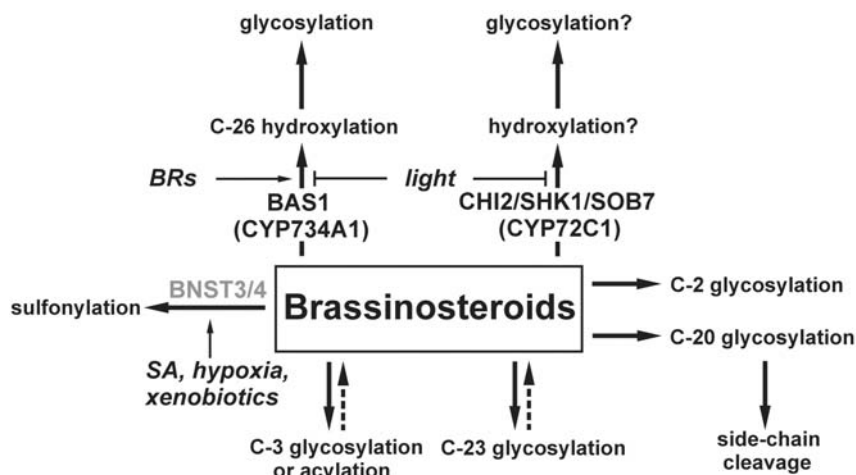
### 3.2.2 Inactivation

Optimal endogenous levels of BRs are maintained by the action of biosynthetic and inactivation enzymes. The biochemical processes of BR inactivation ensure the removal of excess BRs by converting them, reversibly or irreversibly, to biologically inactive forms. Several types of BR metabolites have been identified by the analysis of steroid-containing compounds produced in explants or cell cultures following feeding with isotope-labelled BRs. Suzuki *et al.* (1993) found that in mung bean BL becomes glycosylated at the C-23 hydroxyl group. Upon exogenous application the isolated conjugate showed bioactivity, suggesting that *in planta* BL is released from the conjugate. In *Lilium logiflorum* teasterone was shown to form reversible C-3 acyl-conjugates in a developmentally regulated manner (Asakawa *et al.*, 1996; Soeno *et al.*, 2000). Reversible conjugation at the C-23, C-2 and possibly also the C-3 hydroxyls may play an important regulatory role by allowing temporary removal of bioactive BRs. By contrast, irreversible inactivation takes place by P450-mediated hydroxylation and subsequent glycosylation of BRs at the C-25 and C-26 positions (Hai *et al.*, 1995; 1996). Furthermore, Kolbe *et al.* (1996) reported that hydroxylation at C-20 results in side-chain cleavage, leading to pregnane-type catabolites. The biochemical mechanisms of BR inactivation are summarised in Fig. 3.4. Recently, some BR-inactivating functions have been characterised in *Arabidopsis* and *Brassica napus* using molecular genetic approaches. These are discussed in the following sections.

#### 3.2.2.1 BAS1

Neff *et al.* (1999) characterised an activation-tagged *Arabidopsis* mutant overexpressing the *PHYB4 ACTIVATION-TAGGED SUPPRESSOR 1* (*BAS1*) gene and showing a similar phenotype to those of BR-deficient plants. *BAS1* encodes CYP734A1





**Figure 3.4** Mechanisms of BR inactivation. The BR-inactivating enzymes identified in *Arabidopsis* (black) and *Brassica napus* (grey) are indicated at the arrows representing enzymatic reactions. Possibly reversible reactions are designated by broken arrows.

(formerly CYP72B1), a P450 enzyme that is only distantly related to the CYP85 and CYP90 families involved in BR biosynthesis. A role for BAS1 in the irreversible inactivation of BRs is illustrated by the findings that the *bas1-D* mutant can be rescued by exogenously applied BL, it contains diminished levels of BRs, but accumulates the biologically inactive BR, 26-hydroxy-BL. Furthermore, yeast-expressed CYP734A1 converts bioactive BL and castasterone to their respective C-26-hydroxylated derivatives (Turk *et al.*, 2003). Under physiological conditions *BAS1* is expressed at very low levels, but its activity is strongly induced by BRs (Choe *et al.*, 2001; Goda *et al.*, 2002; Turk *et al.*, 2003).

### 3.2.2.2 *CHI2/SHK1/SOB7*

Another activation-tagged mutant of *Arabidopsis*, *chibi 2* (*chi2*) shows a BR-deficient dwarf phenotype similar to that of *bas1-D* (Nakamura *et al.*, 2005). This dominant mutation results in overexpression of the *CHI2* gene that encodes CYP72C1, a P450 sharing 47% amino acid sequence identity with CYP734A1 (Nakamura *et al.*, 2005). Whereas CHI2/CYP72C1 also utilises castasterone and BL as substrates, the CYP72C1 overexpressing *sob7-D* mutant does not accumulate C-26-hydroxylated BRs, indicating that the inactivation reaction catalysed by this enzyme is different from that of CYP734A1 (Turk *et al.*, 2005). In contrast to *BAS1*, the expression of *CYP72C1* is not induced by BRs (Nakamura *et al.*, 2005; Takahashi *et al.*, 2005). The expression of both *CYP734A1* and *CYP72C1* is light-regulated, and their levels influence photomorphogenesis and the accumulation of bioactive BRs (Turk *et al.*, 2003; 2005). Therefore, these enzymes seem to play an important role in the integration of light and steroid signaling pathways.



### 3.2.2.3 *UGT73C5*

Recently Poppenberger *et al.* (2005) have reported that *UGT73C5*, an *Arabidopsis* UDP-glycosyltransferase, catalyses 23-*O*-glucosylation of BRs. Conversion assays carried out with yeast-expressed *UGT73C5* revealed that BL and castasterone are the preferred substrates of this enzyme. Transgenic overexpression of the *UGT73C5* gene lead to decreased BR content and dwarfness, indicating that 23-*O*-glucosylation abolishes the bioactivity of BRs. In expression analyses, utilising a promoter-GUS reporter fusion, strong *UGT73C5* promoter activity was detected in seedlings, especially in the elongation zones of roots and hypocotyls (Poppenberger *et al.*, 2005).

### 3.2.2.4 *BNST3 and BNST4*

The salicylate-inducible *BNST3* gene of *Brassica napus* encodes a steroid sulphotransferase (SOT) that facilitates the removal of early BR biosynthesis intermediates by sulphonating them at the C-22 hydroxyl. Although sulphonation by *BNST3* could abolish the activity of 24-epi-BL, *in vitro* the enzyme showed preference for 24-epicathasterone (Rouleau *et al.*, 1999). Further characterisation of *BNST3* and the related *BNST4* revealed that in addition to salicylate, the expression of these *Brassica* genes can also be induced by hypoxia, ethanol and various xenobiotics. Overexpression of *BNST3* in *Arabidopsis* does not lead to a BR-deficient phenotype, therefore this enzyme may have a role in controlling the pool size of early BR intermediates. By contrast, *BNST4*, which is capable of sulphonating a broad range of steroid substrates, has been implicated in stress-related detoxification mechanisms (Marsolais *et al.*, 2004).

In *Arabidopsis*, the protein family of SOTs is predicted to comprise 18 members. Although the functions of most of these enzymes is unknown, one of them participates in jasmonate inactivation (Klein & Papenbrock, 2004). It remains to be elucidated whether any of the *Arabidopsis* SOTs are involved in the control of *in planta* BR levels.

## 3.2.3 *Functional aspects of BR metabolism*

### 3.2.3.1 *Regulation of biosynthetic genes*

Several lines of evidence indicate that transcriptional regulation of the BR-biosynthetic genes plays a crucial role in the control of BR synthesis and, hence, in determining the endogenous level of the hormone. It has been shown that the level of *CPD* mRNA is regulated primarily at the transcriptional level (Mathur *et al.*, 1998), and that weak *CPD* expression in the roots of *Arabidopsis* coincides with the accumulation of its proposed substrate, 6-deoxocathasterone (Bancos *et al.*, 2002). Most BR-biosynthetic genes are under complex transcriptional control that involves multiple regulatory mechanisms.

One of these mechanisms is the feedback regulation of all P450-encoding biosynthetic genes by the action of bioactive BRs. In *Arabidopsis* *DWF4*, *CPD*, *ROT3*, *CYP90D1*, *CYP85A1* and *CYP85A2* are co-ordinately down-regulated by bioactive BRs, reducing their transcript level to around 10% of the initial value within 2 h of BL

treatment (Bancos *et al.*, 2002; Goda *et al.*, 2002; Tanaka *et al.*, 2005). Feedback regulation of the *CPD* gene depends on BR signaling initiating at the *BRI1* receptor (Li *et al.*, 2001b; Bancos *et al.*, 2002). It is mediated by the *BZR1* transcription factor that binds as a repressor to a specific BR response element present in the promoter of *CPD* and all the other feedback-controlled genes of BR synthesis (He *et al.*, 2005). In the *bril* BR-insensitive mutant the lack of feedback regulation results in abnormally high levels of BR accumulation (Noguchi *et al.*, 1999a). In wild-type plants, the activity of BR-biosynthetic P450 genes is partially repressed, allowing either up- or down-regulation of their expression through the feedback mechanism at a wide range of endogenous BR concentrations (Bancos *et al.*, 2002). Therefore, the feedback control is believed to be an important physiological regulator of BR synthesis and accumulation (Bancos *et al.*, 2002; Tanaka *et al.*, 2005).

A further level of transcriptional regulation ensures organ-specific expression. In contrast to the ubiquitously expressed *DET2*, the P450 genes of BR biosynthesis show distinct types of organ specificity (Bancos *et al.*, 2002; Tanaka *et al.*, 2005). Spatial regulation of these genes seems to be important in determining the BR levels in different organs. The *CPD* and *CYP85A2* enzymes, which catalyse rate-limiting reactions, are expressed more intensely in shoots than in roots of *Arabidopsis* seedlings (Bancos *et al.*, 2002; Shimada *et al.*, 2003). This is in good agreement with the finding that in *Arabidopsis*, tomato and pea, shoots are richer in 6-deoxocastasterone and bioactive BRs, whereas roots accumulate 6-deoxotyphasterol and farther upstream BR intermediates (Yokota *et al.*, 2001; Bancos *et al.*, 2002). The physiological significance of localised gene expression is highlighted by the reported lack of *in planta* BR transport (Bishop *et al.*, 1996; Symons & Reid, 2004; Montoya *et al.*, 2005), which suggests that the expression sites of rate-limiting biosynthetic enzymes coincide with the sites of active BR synthesis (Castle *et al.*, 2005; Montoya *et al.*, 2005; Nomura *et al.*, 2005).

The transcription of BR-biosynthetic genes is also under developmental regulation. In *Arabidopsis* all *CYP85* and *CYP90* genes are strongly expressed in germinating seeds and young seedlings (Bancos *et al.*, 2002). This is in good agreement with the role of BRs in hypocotyl elongation and vascular differentiation (Cano-Delgado *et al.*, 2004; Castle *et al.*, 2005). Recent reports have shown light-induced accumulation of castasterone and BL in pea seedlings (Symons *et al.*, 2002; Symons & Reid, 2003), indicating the possibility that BR-biosynthetic genes are also activated during de-etiolation. The importance of BRs in photomorphogenic development is highlighted by the de-etiolated dark phenotypes of severely BR-deficient mutants (Chory *et al.*, 1991; Li *et al.*, 1996; Szekeres *et al.*, 1996; Azpiroz *et al.*, 1998).

### 3.2.3.2 Regulation of BR-inactivating genes

Expression levels of BR-inactivating genes are also stringently controlled at the transcriptional level. The gene encoding *BAS1/CYP734A1* is BR inducible (Choe *et al.*, 2001; Turk *et al.*, 2003), indicating that it has a function in the homeostatic regulation of BR content. Choe *et al.* (2001) have shown that this feedforward control requires the *BRI1* receptor. By contrast, the expression of *CYP72C1* is not influenced by BRs

(Takahashi *et al.*, 2005). Expression of the *CYP734A1* and *CYP72C1* genes were found to be down-regulated by light (Turk *et al.*, 2003; Nakamura *et al.*, 2005; Takahashi *et al.*, 2005), although in seedlings grown in continuous dark the level of the *BAS1/CYP734A1* transcript was slightly lower than those in continuous white light (Turk *et al.*, 2005). Light regulation of these genes seems to be closely connected to the transition from skotomorphogenesis to photomorphogenesis, because mutations affecting *CYP734A1* or *CYP72C1* expression result in altered light responsiveness. This notion is further supported by data showing that white, red and blue light repressed, but far-red induced accumulation of the *BAS1/CYP734A1* transcripts and its protein products in the elongation zone of etiolated seedlings (Turk *et al.*, 2003). The expression of the UGT73C5 UDP-glycosyltransferase was also found associated with the elongating tissues of seedlings (Poppenberger *et al.*, 2005). This localisation suggests that the level of BRs needs to be stringently controlled at the sites of BR action.

### 3.2.3.3 Conservation of BR synthesis in higher plants

All currently available information indicates a remarkable conservation of BR biosynthesis between plant species belonging to different families. For example, in *Arabidopsis*, pea and tomato there is an accumulation of high levels of 6-deoxocathasterone and 6-deoxocastasterone, which are indicative of common rate-limiting reactions in the pathway (Nomura *et al.*, 2001). In rice, however, 6-deoxotyphasterol is the most abundant BR intermediate, suggesting that in monocots both C-6 oxidation and C-2 hydroxylation can be rate-limiting conversion steps (Hong *et al.*, 2002; Mori *et al.*, 2002). With the exception of the Solanaceous plants tomato and tobacco, in which no early C-6 oxidation intermediates have been detected (Suzuki *et al.*, 1995; Bishop *et al.*, 1999), other species seem to synthesise BRs through both the early and late C-6 oxidation pathways. In each case, however, the late C-6 oxidation route was found to be predominant. Recently, the functional significance of early C-6 oxidation has become uncertain in the light of data showing 6-oxocampestanol accumulation in the *cyp85a1cyp85a2-1 Arabidopsis* mutant deficient in both late C-6 oxidase enzymes (Kwon *et al.*, 2005).

Shimada *et al.* (2001, 2003) reported that yeast-expressed CYP85A1 and CYP85A2 converted not only 6-deoxocastasterone but also 6-deoxotyphasterol and 6-deoxoteasterone to their respective 6-oxo derivatives. Other BR-biosynthetic enzymes also have relaxed substrate specificities that allow their participation in different sub-pathways (Fig. 3.3) and it therefore seems possible that BRs are synthesised via a network of reactions. This concept is strengthened by the natural occurrence of 3-*epi*-6-deoxocathasterone (Fujioka *et al.*, 2002) and 2-deoxo-BL (Schmidt *et al.*, 1995; Yokota *et al.*, 1996), which indicate that C-3 epimerisation or lactonisation of the B ring may precede the C-23 or C-2 hydroxylation steps, respectively. *In vitro* enzyme kinetic studies are expected to yield valuable information regarding the preferences of DET2 and BR-biosynthetic P450s toward their substrates.

3.3 Signal transduction

At the same time as the advances in our understanding of BR biosynthesis have been made, remarkable progress and insights into BR signaling have occurred. A key feature of this has been the observation that a functional BR signaling mechanism is required for the transcriptional feedback regulation of BR-biosynthesis genes. The following sections reviewing BR-signaling highlight the success of novel genetic screens and the isolation of mutations/genes in the BR-signaling pathway. This genetic approach has been crucial in the rapid advance made, and more recently this has been backed-up with elegant biochemical/molecular data. This synergy of approaches has enabled a detailed description of the function of these proteins and also the discovery of further factors in the signaling pathway. As an aid to the following sections, a table of the key signaling factors has been provided (Table 3.2).

**Table 3.2** Key BR-signal transduction genes in *Arabidopsis*

Genes/alleles	Function	References
<i>BR11</i>	LRR-RLK	Clouse <i>et al.</i> (1996), Li and Chory (1997)
<i>BAK1</i>	LRR-RLK	Li <i>et al.</i> (2002), Nam and Li (2002)
<i>BIN2</i> , <i>DWF12</i> , <i>UCU1</i>	GSK3 like kinase	Li <i>et al.</i> (2001b), Choe <i>et al.</i> (2002), Li and Nam (2002), Pérez-Pérez <i>et al.</i> (2002)
<i>BSU1</i>	Kelch-repeat-containing phosphatase	Mora-Garcia <i>et al.</i> (2004)
<i>BZR1</i>	Transcription factor	Wang <i>et al.</i> (2002)
<i>BES1</i>	Transcription factor	Yin <i>et al.</i> (2002)
<i>BIM1</i>	bHLH transcription factor	Yin <i>et al.</i> (2005)
<i>BR51</i>	Serine carboxypeptidase	Li <i>et al.</i> (2001a)

3.3.1 *BR11* and *BAK1*

When BRs are applied to plants a signaling cascade is initiated at the plasma membrane where BRs bind to a BR receptor, BRASSINOSTEROID INSENSITIVE 1 (BR11). *BR11* encodes a leucine rich repeat receptor-like kinase (LRR-RLK) (Li & Chory, 1997) and mutants in this gene were first identified in genetic screens for plants exhibiting dwarfism and lacking inhibition to root growth in the presence of BRs (Clouse *et al.*, 1996). Many experiments have highlighted the importance of BR11 in BR signaling which has culminated in the recent discovery that BRs bind directly to BR11 (Kinoshita *et al.*, 2005) and this leads to a change in state from an autoinhibitory form to a more active kinase (Wang *et al.*, 2005b). BR11 is not the only BR receptor, as other LRR-RLKs similar to BR11 have been identified that also interact with radio-labelled BRs (Cano-Delgado *et al.*, 2004). BRs stimulate autophosphorylation of BR11, and the phosphorylation status of BR11 is also modulated via the interaction with another LRR-RLK BRASSINOSTEROID-ASSOCIATED KINASE 1 (BAK1) (Wang, G.-T. *et al.*, 2005). BAK1 was identified based on its ability to suppress the

*brl1* dwarfing phenotype when over-expressed, and via a yeast 2-hybrid screen (Li *et al.*, 2002; Nam & Li, 2002). It is likely that as in the proposed activation mechanism of animal receptor kinase signaling, BRs promote BRI1 and BAK1 to form an activated receptor complex via the change in phosphorylation status of this complex.

Potential downstream targets of BRI1/BAK1 have been identified, although their exact role in the BR signaling process has yet to be clarified. TRIP-1 (transforming growth factor beta (TGF $\beta$ )), a receptor interacting protein has been shown to co-immunoprecipitate with BRI1, and it has been demonstrated that the BRI1 kinase can phosphorylate TRIP1 (Ehsan *et al.*, 2005). Antisense TRIP1 plants also exhibit dwarfism and other phenotypic similarities to those of BR-signaling mutants, suggesting that TRIP1 is involved in the BR response. It remains to be clarified how TRIP1, a component of the eIF3 translation initiation factor, functions in BR signaling.

A yeast 2-hybrid screen for other BRI1 substrates using the BRI1 kinase domain as bait has identified a transthyretin-like (TTL) protein (Nam & Li, 2004). *In vitro* experiments indicate that BRI1 phosphorylates TTL, and genetic evidence suggests that TTL is a negative regulator of BR-induced plant growth (Nam & Li, 2004). The co-expression of TTL with BRI1, and their localisation in the plasma membrane provides further evidence that TTL has a role in BR signaling. The mechanism, however, by which TTL mediates responses toward downstream signaling components remains a mystery.

### 3.3.2 *BIN2 and BSU1*

Genetic screens for mutants insensitive to BRs mainly identified recessive mutations in *BRI1*. However, careful re-examination of the mutants obtained from such screens also led to the identification of the *BR INSENSITIVE 2* locus (*BIN2*). *BIN2* was isolated by map-based cloning and shown to have homology to mammalian Glycogen Synthase Kinase 3- $\beta$  (GSK3) and the Shaggy protein kinase of *Drosophila* (Li *et al.*, 2001b; Li & Nam, 2002). *BIN2* is one member of an *Arabidopsis* gene family comprised of ten genes. In the *bin2* gain-of-function mutants, increased *BIN2* activity resulted in dwarf plants. Genetic and molecular data indicated that *BIN2* was acting as a negative regulator of BR synthesis, such that down-regulation of *BIN2* activity is required for BR-mediated responses. An additional mutant at the *BIN2* locus (*UCU1*) was identified at the same time via screens for altered leaf morphology (Pérez-Pérez *et al.*, 2002).

As mentioned earlier, activation tagging in the *brl1* mutant background led to the identification of the *bak1* mutation. A similar screen led to the isolation of an additional suppressor mutant, *bsu1* (*brl1* suppressor 1) (Mora-Garcia *et al.*, 2004). *BSU1* encodes an apparently plant-specific phosphatase with N-terminal Kelch-repeat and C-terminal S/T phosphatase domains. The *bsu1* mutation partially suppresses the *bin2* mutant phenotype, and *bsu1* RNAi lines exhibit a phenotype similar to the weak alleles of *brl1*. These results suggest that the *BSU1* phosphatase acts antagonistically to the *BIN2* kinase activity. This antagonism is apparent in the phosphorylation status of the *BIN2/BSU1* substrates, BZR1 and BZR2/BES1, as discussed in the next section.

### 3.3.3 *BZR1* and *BZR2/BES1*

Brassinazole (BRZ) is a triazole-type inhibitor of BR synthesis developed to specifically interact with the P450 enzymes of the pathway (Asami & Yoshida, 1999; Asami *et al.*, 2000). This inhibitor, when applied at  $\mu\text{M}$  to  $\text{mM}$  concentrations, has been shown to generate dwarfs that are phenotypically similar to the BR-biosynthetic and BR-signaling mutants (Asami & Yoshida, 1999; Asami *et al.*, 2000). To identify further BR-signaling components mutations that suppress the inhibitor-induced dwarfism in the dark were identified, namely the brassinazole-resistant *bzr1* and *bzr2* genes (Wang *et al.*, 2002). The *bes1* (*brl1* *ems* *suppressor 1*) mutation was identified in a genetic screen for suppressors of the *brl1* mutant phenotype (Yin *et al.*, 2002) and was found to be allelic to *bzr2*. *BZR1* and *BES1* are highly homologous and are part of a plant-specific gene family that in *Arabidopsis* has six members. The *bzr1* and *bes1* mutations are gain-of-function mutations that both lead to a P to L substitution in the proteolysis-linked PEST domain. Although *bzr1* and *bes1* have similar mutations in their protein sequence and exhibit similar dark-grown phenotypes, their light-grown phenotypes are different. The *bzr1* mutants have a weak dwarf phenotype and increased sensitivity to BRZ, whereas the *bes1* mutants have a phenotype similar to lines over-expressing *BRI1*. This suggested that *BZR1* acts as a transcriptional repressor, whereas *BES1* as an activator.

The *BZR1* and *BZR2/BES1* proteins have nuclear localisation sequences, and when fused to FP (fluorescent protein) targeting of these proteins to the nucleus is observed. *BZR1* and *BES1* over-expression suppresses the *bin2* phenotype, indicating that *BZR1* and *BES1* act downstream of *BIN2*. *In vitro* experiments have shown that *BIN2* can phosphorylate *BZR1* and *BES1*, and that in the *bin2* dominant dwarf lines the levels of *BZR1* and *BES1* are reduced (He *et al.*, 2002). The proteasome inhibitor MG132 allowed the accumulation of phosphorylated *BZR1*, indicating that phosphorylation of *BZR1* targets it for degradation (He *et al.*, 2002). More recent has been the observation that *BZR1* and *BES1* bind to DNA in distinct manners, with *BZR1* binding to upstream regulatory sequences of BR down-regulated (BRD) genes, whereas *BES1* binds to sequences upstream of BR up-regulated (BRU) genes. *BZR1* is therefore a key component in the feedback regulation of BR synthesis, which is consistent with the observed difference between the light-grown phenotypes of the *bzr1* and *bes1* mutants (He *et al.*, 2005).

### 3.3.4 *BIM1*

The discovery that *BZR1* and *BES1* have DNA binding activity was made at the same time as that of the bHLH transcription factor *BIM1* (*BES* *I**NTERACTING* *MYC*-*L**I**K**E* *1*) (Yin *et al.*, 2005). *BIM1* was identified by screening for proteins that interact with the *BES1* C-terminal domain. The triple mutant defective in *BIM1* and those in its close homologues *BIM2* and *BIM3* is dwarfed under light and dark conditions. The over-expression of *BIM1* can partially suppress the weak *brl1* dwarf phenotype and this causes reduced sensitivity to BRZ. By contrast, the triple mutant is more sensitive



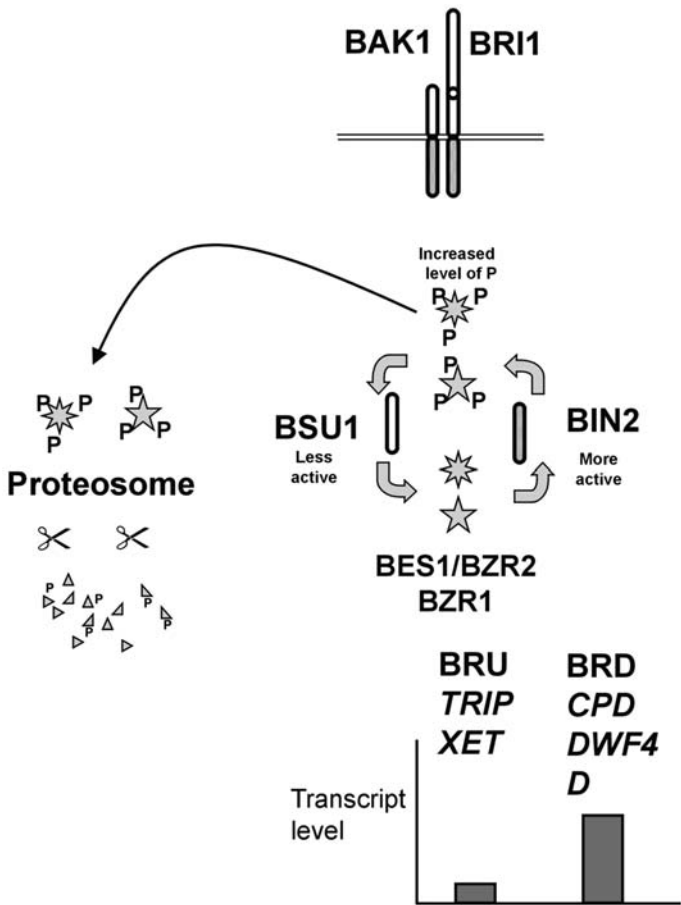
to the inhibitor (Yin *et al.*, 2005). These data highlight the fact that BIM1, BIM2 and BIM3 are involved in BR response.

Binding assays of BES1 and BIM1 to target gene promoters have shown specific binding to the E-box sequence motif (CANNTG), and a model has been presented suggesting that binding occurs in a heterodimer form (Yin *et al.*, 2005). BZR1 also acts as a transcription factor that binds to the CGTG(T/C)G BR-response element (BRRE) (He *et al.*, 2005). Analysis of microarray data and targets for BZR1 suggests that this transcription factor acts as a repressor that is important both in BR homeostasis and growth responses (He *et al.*, 2005). It is not yet known, however, if BZR1 forms a heterodimer with a yet to be identified transcription factor.

### 3.3.5 Signaling mechanism and other putative components

Recent models and reviews of BR signaling have been given by Vert *et al.* (2005), Li (2005) and Li and Deng (2005). Here we summarise these models by showing the process of BR signaling in the absence (Fig. 3.5) or presence of BRs (Fig. 3.6). When BRs are absent, the BRI1 receptor and its co-receptor BAK1 are not activated and this state of the receptor complex does not reduce the activity of BIN2 kinase, or increase the activity of BSU1 phosphatase. BIN2 phosphorylates BES1 and BZR1, and the phosphorylated forms are targeted for degradation in the proteasome. This leads to a reduced level of hypophosphorylated BES1 and BZR1 in the nucleus that causes reduced expression of BRU genes and increased expression of BRD genes, for example the BR-biosynthesis genes *DWF4* and *CPD* (Fig. 3.5). Conversely, in the presence of BRs, BR binds to BRI1 leading to phosphorylation of the BRI1 and BAK1 kinase domains in the receptor complex. By some unknown mechanism, reduction in the activity of the BIN2 kinase and/or increase of BSU1 phosphatase activity occurs. This results in an increased concentration of hypophosphorylated BES1 and BZR1 in the nucleus. BES1 with BIM2 bind to E-box sequences to up-regulate a subset of BR responsive genes. BZR1 binds to BRRE sequences and acts as a repressor, that is feedback regulator, for BR-biosynthesis genes (e.g. *DWF4* and *CPD*) and other BR down-regulated genes (Fig. 3.6).

The initial activation of BRI1 is somewhat different from that suggested before, whereby BRs were thought to bind to proteolytically processed sterol binding proteins (SBPs), and these BR-protein complexes were to interact with the LRRs of BRI1 to activate a kinase cascade (as reviewed by Bishop & Koncz, 2002). Initial support for the SBP involvement in BR signaling was provided via the isolation of a dominant mutant obtained by activation tagging in the weak *bri1-5* background. BRS1 (BRI1 SUPPRESSOR 1), a carboxypeptidase, was found to act upstream of BRI1 (Li *et al.*, 2001a). Li *et al.* (2001b) postulated that a SBP could be a potential substrate for BRS1. Recently there has been further evidence to suggest a role for SBPs in BR signaling, showing that over-expression of MSBP1 (MEMBRANE STEROL BINDING PROTEIN 1) reduces, whereas its under-expression increases growth (Yang, X.H. *et al.*, 2005). This alteration in SBP levels, however, did not affect the expression of BR-biosynthetic genes. Further studies into how BR-signaling processes are affected by



**Figure 3.5** Model showing the status of the BR signaling components in the absence of BRs. In the absence of BRs the BRI1/BAK1 leucine-rich repeat receptor kinases are not activated. The BIN2 kinase activity is increased and/or the BSU1 phosphatase activity is decreased by some unknown mechanism. This leads to increased phosphorylation of the transcription factors BZR1 and BES1, which activates their degradation by the proteasome. The lack of these transcription factors in the nucleus prevents the transcription of BR up-regulated genes (BRU), and the repression of BR down-regulated genes (BRD).

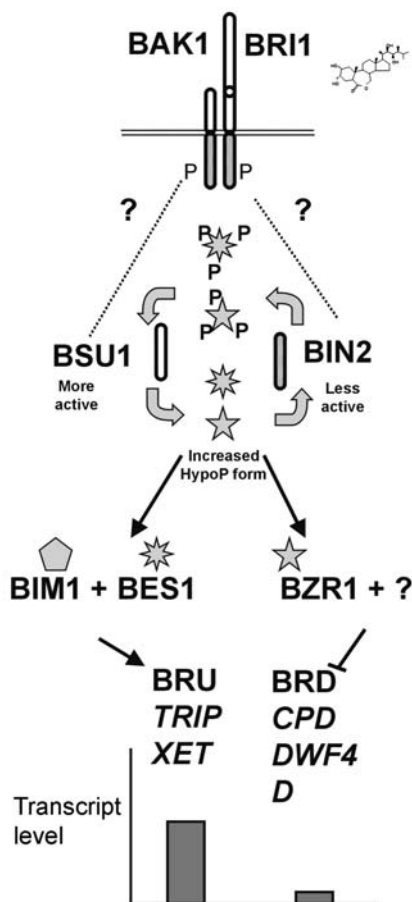
BRS1 and MSBP-lines with altered expression will be required to discern the role, if any, of SBPs and BRS in BR-mediated signaling.

3.4 Future prospectives

3.4.1 Metabolism

The reactions of BR biosynthesis are relatively well known, however the significance of known or yet unknown sub-pathways is yet to be clarified. BRs are synthesised in





**Figure 3.6** Model showing the status of the BR signaling components in the presence of BRs. In the presence of BRs, binding of the hormone to BRI1 leads to activation of the BRI1/BAK1 kinases. The BIN2 kinase activity is decreased and/or the BSU1 phosphatase activity is increased by some unknown mechanism. This leads to hypo-phosphorylation of the transcription factors BZR1 and BES1, which leads to their increased concentrations in the nucleus. BES1 and BIM1, an additional transcription factor, promote the transcription of BR up-regulated genes (BRU). BZR1, and possibly unknown transcription factor(s), binds to the upstream regulatory regions of BR down-regulated genes (BRD) leading to their reduced transcription.

a complex pathway, and in biosynthetic mutant plants conversion rates and intermediate levels are distorted by the hormonal regulation of both biosynthetic and catabolic enzymes. Therefore, BR analysis and intermediate rescue data cannot always give conclusive information regarding the enzymatic functions affected in biosynthetic mutants. Recent progress in heterologous expression of functional CYP85s and CYP90s, and their enzymological characterisation seems to be instrumental in understanding the substrate preferences of these P450s. Because *in vitro* conditions

may influence enzymatic functions, this promising technique can be most powerful when used in combination with the conventional molecular genetic and analytical methods.

Detailed characterisation of BR biosynthesis and signaling will greatly benefit from the development of efficient, less time consuming methods capable of quantitating BR levels in different organs and tissues. Such techniques will be helpful in elucidating if/how organ-specific expression of biosynthetic genes can influence local accumulation of BRs. Future studies are needed to clarify whether any of the BRs or their conjugates are transported within the plant. Current evidence suggests that bioactive BRs act in paracrine/autocrine manner, with very limited transport between the tissues and organs (Symons & Reid, 2004; Montoya *et al.*, 2005).

### 3.4.2 *Signal transduction*

Of key importance in BR signaling will be to determine the signaling process by which the activated BRI1/BAK1 kinases lead to the down-regulation of BIN2 kinase activity, and/or increased BSU1 phosphatase activity. Similarly, clarification of the regulation and mechanism by which the receptor complex becomes deactivated will be of great interest. Endocytosis of the BRI1/BAK1 complex has been observed (Russeinova *et al.*, 2004), and this offers an enticing mechanism by which either of the processes above may occur. Subcellular imaging of where the signaling events take place will also be an exciting area from which greater understanding of the BR-signaling process will be gained. One fascinating problem is whether the BIN2 kinase can gain access to the nucleus and deactivate the BES1/BZR1 transcription factors. There are numerous other lines of investigation that will provide highly informative and novel results, for example by determining the structure of the receptor-BR complex, or defining the roles of TTL, TRIP1, BRS1 and MSBP1 in the BR-signaling process.

### 3.4.3 *Crops*

It will be prudent to utilise our knowledge of BR metabolism and signaling to improve crop performance. Conceivably, by altering these process in crops increased yield will be possible through increased biomass or indirectly through greater resistance to stress factors, for example wind damage (lodging) and elevated temperatures. Many advances in our understanding of BR biosynthesis and signaling have been made in barley, cotton, pea, rice and tomato, through the isolation of BR-related mutants and identification of the genes affected by these mutations. Further advances in this area will enable better understanding of the conservation of BR related processes and provide methodologies for crop improvement. The adoption therefore of novel breeding strategies, using transgenic or non-transgenic means, are set to yield the desired fruits of BR research.

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## 4 Cytokinin metabolism and signal transduction

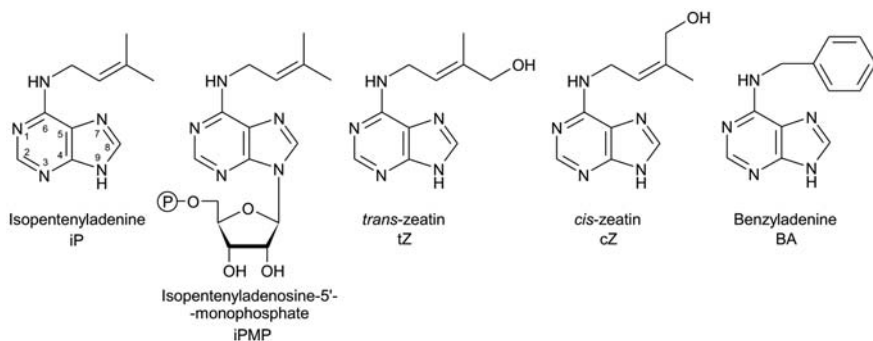
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### 4.1 Introduction

Cytokinin plays an important role in many physiological and developmental processes in the plant, such as regulation of shoot and root growth, leaf senescence, chloroplast development, stress response and pathogen resistance (Mok & Mok, 2001). Recent years have witnessed rapid progress in our understanding of cytokinin metabolism. This topic will be addressed first in this review. In the second part, we will describe the perception and transduction of the cytokinin signal, which was also elucidated at the beginning of this century.

### 4.2 Cytokinin metabolism

The most abundant naturally occurring cytokinins are adenine derivatives substituted at the N<sup>6</sup>-position with an isoprenoid side chain (Fig. 4.1). In addition, cytokinin molecules carrying an aromatic side chain were detected in several plant species and usually display a high biological activity in different bioassays (Strnad, 1997). These so-called aromatic cytokinins bind to cytokinin receptors and activate signaling (Spichal *et al.*, 2004; see also below). The biosynthetic pathway leading to aromatic cytokinins is, however, entirely elusive. Thus, the recent elucidation of cytokinin biosynthesis, conjugation and catabolism concerns exclusively isoprenoid-type cytokinins.



**Figure 4.1** Chemical structures of some naturally occurring cytokinins carrying an isoprenoid or aromatic side chain.

Cytokinin metabolism is a dynamic balance between biosynthesis, interconversion, the formation of conjugates, which may retain a certain degree of cytokinin activity, and the catabolic reaction that results in a loss of biological activity. Evidence is accumulating that in the plant tissue the local cytokinin concentration is a key factor that determines the strength of the physiological response. Therefore, an important question is how the physiologically optimal cytokinin concentration is achieved in a spatially and temporally distinct manner.

#### 4.2.1 Cytokinin biosynthesis

Earlier studies on ipt (isopentenyl diphosphate transferase) enzyme activity in *Dictyostelium discoideum* (Taya *et al.*, 1978) and of the T-DNA-encoded tmr/ipt enzyme of *Agrobacterium tumefaciens* (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984) had shown that the first and rate-limiting step of *de novo* cytokinin biosynthesis is the transfer of the isopentenyl group from dimethylallyl diphosphate (DMAPP) to the N<sup>6</sup>-position of adenosine monophosphate (AMP) (Fig. 4.2). This leads to the formation of isopentenyladenosine-5'-monophosphate (iPMP). Such a mechanism was anticipated also for the IPT enzymes of plants. In *Arabidopsis*, seven genes coding for IPT enzymes were identified (*AtIPT1*, *AtIPT3*–*AtIPT8*). It was demonstrated that the *AtIPT* enzymes have the capacity to produce isopentenyladenine (iP) and *trans*-zeatin (tZ) when expressed in *E. coli* (Kakimoto, 2001; Takei *et al.*, 2001a). Additionally, the overexpression of *AtIPT4* and *AtIPT8* in *Arabidopsis* resulted in cytokinin-independent growth (Kakimoto, 2001; Sun *et al.*, 2003). Detailed biochemical studies revealed that, unlike the agrobacterial enzymes, purified recombinant *AtIPT* proteins utilize preferentially adenosine-5'-diphosphate and adenosine-5'-triphosphate (ADP and ATP) rather than AMP as a substrate (Fig. 4.2) (Kakimoto, 2001; Takei *et al.*, 2003). This unexpected result suggests that the initial products of cytokinin biosynthesis *in planta* are isopentenyladenosine-5'-diphosphate (iPDP) and/or -triphosphate (iPTP), respectively. Considering that the most active form of cytokinin is its base, the initially synthesized cytokinin nucleotides will be subsequently dephosphorylated and deribosylated.

In plants, two distinct biosynthetic pathways supply the common isoprenoid precursors isopentenyl diphosphate (IPP) and its isomer DMAPP, which is recruited through the ipt-catalyzed reaction into the side chain of cytokinin. The classical mevalonate (MVA) pathway usually occurs in the cytosol of eukaryotes; the alternative methylerythritol phosphate (MEP) pathway (also known as 1-deoxy-D-xylulose 5-phosphate pathway, DXP) was discovered as typical for bacteria and plastids of plants (Lichtenthaler, 1999). The relative contributions of these pathways to cytokinin biosynthesis were recently addressed in a study employing feeding of <sup>13</sup>C-labelled metabolic precursors in *Arabidopsis* mutant plants defective in the MEP pathway or in wild-type plants with a chemically blocked MVA pathway (Kasahara *et al.*, 2004). It was shown that tZ- and iP-type cytokinins in *Arabidopsis* seedlings are predominantly synthesized via the plastidic MEP pathway, whereas



*et al.*, 2004). The hypothesis that the majority of tZ- and iP-cytokinins have a plastidic origin is further supported by the notion that the endogenous cytokinin concentration was not altered in *hmg1 Arabidopsis* mutants, which have a defect in the first step of the cytosolic MVA pathway (Suzuki *et al.*, 2004).

The tZ-type cytokinins can either be derived from iP-type cytokinins by hydroxylation of the side chain (Fig. 4.1), which could occur at different steps during synthesis of iP-type cytokinins, or, alternatively, an already hydroxylated precursor molecule could be fused instead of DMAPP with the adenyl moiety. Several recent experiments suggest that both sorts of metabolic pathways are realized. One is the so-called iPMP-dependent pathway, in which the side chain of the initially synthesized iP-cytokinin is *trans*-hydroxylated by cytochrome P450 monooxygenases. Takei *et al.* (2004a) screened the large cytochrome P450 family of *Arabidopsis* for cytokinin-hydroxylation activity and identified two genes (*CYP735A1* and *CYP735A2*), whose co-expression with *AtIPT4* in yeast resulted in excretion of tZ-cytokinins. It was further shown that CYP735A enzymes utilize in a NADPH-dependent manner iP-nucleotides rather than the nucleosides or free bases as a substrate (Takei *et al.*, 2004b). This indicates that the hydroxylation step occurs at an early stage of cytokinin synthesis, prior to the dephosphorylation of iP-nucleotides (Fig. 4.2). The evidence for an alternative, iPMP-independent, biosynthetic pathway for tZ-cytokinins stems from *in vivo* deuterium labelling experiments with *ipt*-expressing and wild-type *Arabidopsis* plants (Åstot *et al.*, 2000). According to the proposed model, tZ-5'-monophosphate (tZMP) is directly synthesized by *ipt* using an as-yet unknown hydroxylated side-chain precursor. A plausible candidate molecule is hydroxymethylbutenyl diphosphate (HMBPP), which was identified as an intermediate metabolite of the MEP pathway (Hecht *et al.*, 2001). It was indeed shown that purified tzs (*trans*-zeatin secretion) protein of *A. tumefaciens*, which is yet another type of agrobacterial *ipt* protein, catalyzed the transfer of a hydroxylated side chain from HMBPP to AMP, producing tZMP (Krall *et al.*, 2002). Similarly, *in planta* experiments revealed iPMP-independent synthesis of tZ-cytokinins in transgenic plants expressing the agrobacterial *ipt/tmr* gene (Sakakibara *et al.*, 2005). Interestingly, despite the lack of an obvious chloroplast targeting sequence, the *ipt/tmr* protein was found to be localized to the stroma of plastids. However, Sakakibara *et al.* (2005) were unable to demonstrate any utilization of HMBPP in wild-type or *AtIPT1*-expressing *Arabidopsis* plants, which is in conflict with the previous findings (Åstot *et al.*, 2000; Nordström *et al.*, 2004). Instead, it was hypothesized that a portion of tZ-type cytokinins may be produced through the isomerization of possibly tRNA-derived cZ-derivatives (see Fig. 4.2) (Sakakibara *et al.*, 2005). Taken together, in plants the iPMP-dependent pathway is probably predominant for the synthesis of tZ-type cytokinins, while alternative pathways involving hydroxylated precursor molecules or a pathway via cZ await further clarification.

The apparent sites of cytokinin biosynthesis were revealed by the analysis of expression patterns of *AtIPT* gene promoter-*GUS* fusions in *Arabidopsis* (Miyawaki *et al.*, 2004). The peak expression of individual *AtIPT* genes is localized to very distinct domains in the shoot and root, for example axillary buds (*AtIPT1*), young

stems (*AtIPT5*), seeds (*AtIPT1*, -4, -8), trichomes (*AtIPT7*), pollen tubes (*AtIPT7*), phloem (*AtIPT3*), root procambium (*AtIPT1*), columella cells (*AtIPT5*) and lateral root primordia (*AtIPT5*). These results support the view that cytokinins are produced in a wide range of organs and cell types. In accordance, Nordström *et al.* (2004) clearly demonstrated biochemically that both root and shoot tissues possess cytokinin biosynthetic capacity. Several of the *AtIPT* transcripts are down-regulated by cytokinin treatment, indicating negative feedback regulation by the metabolic product (Miyawaki *et al.*, 2004). However, this seems to be a rather slow process as *AtIPT* genes were not identified among the immediate-early response genes of *Arabidopsis* (Rashotte *et al.*, 2003; Brenner *et al.*, 2005). Auxin up-regulates the *AtIPT5* and *AtIPT7* transcripts (Miyawaki *et al.*, 2004). This finding contrasts with other results showing that auxin mediates down-regulation of endogenous cytokinin levels by reducing rapidly the biosynthetic rate of tZMP (Nordström *et al.*, 2004).

An important future task is to elucidate the *in planta* roles of individual *ipt* genes and several recent studies have started to shed light on this. It had been observed previously in diverse plant species that the cytokinin concentration increases upon application of nitrogen-containing minerals and it has been proposed that cytokinin serves as root-to-shoot signal communicating the nitrogen-nutrient status of the root (Singh *et al.*, 1992; Samuelson & Larsson, 1993; Takei *et al.*, 2001b; Sakakibara, 2003). Further analysis indicated that *AtIPT3* is a key determinant of cytokinin biosynthesis in response to rapid changes in the availability of nitrate. Importantly, expression of the phloem-localized *AtIPT3* gene is rapidly up-regulated by nitrate, independently of *de novo* protein synthesis and nitrate-induced cytokinin accumulation was diminished in an *ipt3* knockout mutant plant (Miyawaki *et al.*, 2004; Takei *et al.*, 2004a).

Two independent studies brought exciting evidence that class-I KNOTTED1-like homeobox (KNOX) transcription factors promote shoot apical meristem (SAM) activity through the co-ordinated regulation of high cytokinin–low gibberellin content (Jasinski *et al.*, 2005; Yanai *et al.*, 2005). KNOX proteins were shown to stimulate cytokinin biosynthesis via transcriptional activation of *AtIPT7* (and to a lesser extend *AtIPT5*). The resulting cytokinin activity was in turn both necessary and sufficient to activate the catabolism of gibberellin. In agreement with a reduction of the GA content and/or GA signaling by cytokinin is the additional finding that cytokinin in *Arabidopsis* rapidly suppresses transcripts of GA synthesis genes (*AtGA20ox2* and *AtGA3ox1*) and up-regulates transcripts of negative regulators of the GA signaling pathway (*RGA*, *GAI*) (Brenner *et al.*, 2005). These results are important steps towards a better understanding of the molecular links between cytokinin, other hormones and SAM-specifying transcriptional factors in the shoot apex.

#### 4.2.2 Cytokinin interconversion and conjugation

Interconversions between cytokinin-free bases, nucleosides and nucleotides are major features of cytokinin metabolism. However, these conversions are primarily

related to general purine metabolism, catalysed by enzymes with usually lower affinity for cytokinin compounds than for their adenylyl analogues (Mok & Mok, 2001). A more specific modification of cytokinin compounds is glycosylation. The adenine ring can be glucosylated at different N atoms, which usually results in loss of cytokinin activity. The *in vivo* function of N-glucosylation is unknown but is generally interpreted as one possible inactivation pathway (Mok & Mok, 2001). Hou *et al.* (2004) screened over 100 recombinant glycosyl transferase enzymes (UGTs) of *Arabidopsis* and identified two proteins (UGT76C1 and UGT76C2) able to glucosylate different cytokinins at the N<sup>7</sup> and N<sup>9</sup> positions. Overproduced UGT76C1 was capable of conjugating exogenously applied cytokinin in transgenic *Arabidopsis* plants, but neither the endogenous cytokinin levels nor the plant phenotype were altered (Hou *et al.*, 2004). Therefore, the physiological relevance of these genes is unclear at present.

O-glycosyl conjugates of the N<sup>6</sup> side chain are frequent metabolites in higher plants. Cytokinin O-glycosides have usually low activity in bioassays and are resistant to degradation by cytokinin oxidase/dehydrogenase enzymes (Jameson, 1994). As O-glycosides can be converted back to active cytokinins by  $\beta$ -glucosidases they are believed to represent a cytokinin storage form (Brzobohaty *et al.*, 1993). However, the significance of the O-glycoside salvage for regulating the active cytokinin pool has not been fully understood. Four genes encoding enzymes catalyzing the formation of O-glycosides were characterized in some detail. Zeatin O-glucosyl transferase (ZOG1) and zeatin O-xylosyl transferase (ZOX1) from *Phaseolus* spp. were found to utilize specifically UDP-Glc and UDP-Xyl, respectively, as substrates (Martin *et al.*, 1999a & b). Transgenic tobacco plants constitutively overexpressing ZOG1 had a strongly enhanced concentration of endogenous zeatin O-glucoside, but the levels of other cytokinins including zeatin, the substrate of ZOG1, were not affected (Martin *et al.*, 2001a). The plants were, however, less sensitive to exogenous cytokinin and displayed altered growth, such as a stunted and branched shoot and the enhanced formation of adventitious roots. It remained unclear whether the phenotypic changes were due to enhanced sequestering of active cytokinin or to enhanced levels of metabolically utilizable O-glucosides (Martin *et al.*, 2001a). Recently, two novel genes, *cisZOG1* and *cisZOG2*, encoding O-glucosyl transferases specific for cZ, were isolated from maize (Martin *et al.*, 2001b; Veach *et al.*, 2003). Intriguingly, cytokinin receptors from maize were able to respond to cZ, strongly suggesting a relevant role for cZ in this plant species (Yonekura-Sakakibara *et al.*, 2004). Three cytokinin O-glucosyl transferase genes from *Arabidopsis* were identified, but their *in planta* functions were not yet studied (Hou *et al.*, 2004).

#### 4.2.3 Cytokinin catabolism

Cytokinin with unsaturated isoprenoid side chains are selectively degraded by cytokinin oxidases/dehydrogenases (CKXs) (Mok & Mok, 2001). CKX-mediated irreversible degradation is apparently a major pathway to reduce the pool of active cytokinin and has a significant role in the regulation of cytokinin-dependent developmental processes.



CKX proteins are encoded by small multi-gene families, which were identified in monocots, dicots and lower plants. CKX-like sequences are recognized also in few prokaryotes, for example cyanobacterium (*Nostoc* sp. PCC 7120) and *Rhodococcus fascians* (Schmülling *et al.*, 2003). The *Arabidopsis* genome codes for seven CKX genes (*AtCKX1–AtCKX7*) and analysis of the entire rice genome revealed at least eleven CKX homologues (*OsCKX1–OsCKX11*) (Bilyeu *et al.*, 2001; Schmülling *et al.*, 2003).

CKX enzymes are flavoproteins belonging to a superfamily of FAD-dependent oxidoreductases and it was shown that the FAD cofactor is bound covalently to the recombinant ZmCKX1 apoprotein (Bilyeu *et al.*, 2001; Malito *et al.*, 2004). Several studies demonstrated that oxygen is a poor electron acceptor, and that CKX activity can be greatly enhanced by a variety of artificial electron acceptors under anaerobic conditions (Galuszka *et al.*, 2001; Laskey *et al.*, 2003). This suggests that dehydrogenation is a more likely reaction mode than oxidation (Galuszka *et al.*, 2001). Additionally, the experimentally determined redox potential of the covalently linked flavin indicated *p*-quinone or a similar compound as the most plausible candidate for a physiological electron acceptor of CKX (Frebortová *et al.*, 2004). The three-dimensional structure of recombinant ZmCKX1 protein revealed that the imine form of iP binds with the *isopentenyl* side chain inside an ellipsoidal cavity, while the adenine ring is bound in a funnel-shaped site on the protein surface, thus being accessible to the solvent. Two amino acid residues in the active site, Asp169 and Glu288, seem to be essential for both the specificity of cytokinin binding and enzyme action (Malito *et al.*, 2004).

Analysis of the biochemical properties of several CKX proteins has shown that cytokinin bases and nucleosides, but not nucleotides are the preferred substrates of CKX, which converts them to adenine or adenosine and the corresponding side-chain aldehyde (Fig. 4.2) (Bilyeu *et al.*, 2001; Galuszka *et al.*, 2001). Individual CKX proteins display their maximum reaction rate at different pH (Galuszka *et al.*, 2004), which may reflect the differential subcellular localization and/or glycosylation status of CKX enzymes. Sequence analysis and experimental data showed that the *AtCKX* proteins can be targeted either to the vacuole (*AtCKX1* and *AtCKX3*), the endoplasmic reticulum/extracellular space (*AtCKX2*, *ZmCKX1*) or presumably to the cytoplasm (*AtCKX7*) (Bilyeu *et al.*, 2001; Houba-Herlin *et al.*, 1999; Morris *et al.*, 1999; Werner *et al.*, 2003). Apoplastic CKX enzymes may regulate the concentration of cytokinins available for activation of the presumably plasma membrane-localized cytokinin receptors. CKX enzymes are glycoproteins and it was shown in tobacco cell culture experiments that cytokinin causes an increase of CKX enzymatic activity, of which the majority is associated with an extracellular glycosylated enzyme form (Motyka *et al.*, 2003). It is tempting to speculate that, as part of a regulatory loop, an extracellular cytokinin signal triggers the release of CKX proteins into the apoplast.

Functional specificity of individual CKX genes may be realized at least partly on the level of transcript regulation. *AtCKX* promoter-*GUS* fusion analyses have shown that, similar to the *AtIPT* genes, the maximum expression of individual *AtCKX*

genes was confined to very distinct domains within the shoot and root (Werner *et al.*, 2003). For instance, the expression was localized to the shoot meristematic region (*AtCKX1* and *AtCKX2*), axillary buds (*AtCKX5*), stomata cells (*AtCKX4*, *AtCKX6*), trichomes (*AtCKX4*), developing stamen and pollen (*AtCKX5*), vascular tissue (*AtCKX6*), the root cap (*AtCKX4*) and the procambial region of the root meristem (*AtCKX5*). *ZmCKX1* expression was localized to the vasculature of kernels, roots and coleoptiles of maize and was induced by abscisic acid (ABA) and abiotic stress (Brugiére *et al.*, 2003). Several *AtCKX* genes were found to be up-regulated by cytokinin, indicating substrate control of gene expression. (Brugiére *et al.*, 2003; Rashotte *et al.*, 2003; Brenner *et al.*, 2005; Kiba *et al.*, 2005; Werner *et al.*, 2006). Intriguingly, some *AtCKX* genes are expressed in the same tissue or in close proximity to tissue that expresses cytokinin biosynthesis genes (e.g. vascular tissue, trichomes, axillary buds, columella cells), suggesting that cytokinins are degraded, at least in some instances, where they are synthesized and thus may fulfil their function locally.

Preliminary analysis of loss-of-function *Arabidopsis* mutant plants indicates a high degree of functional redundancy among individual members of the *AtCKX* gene family, because insertional mutation of single *AtCKX* genes causes no or only subtle phenotypic changes (Bartrina I., personal communication). In contrast, it was recently shown that altered expression of a single *CKX* gene in rice can lead to profound alterations of plant development (Ashikari *et al.*, 2005). Ashikari and co-workers mapped quantitative trait loci (QTLs) for grain production in rice and showed that the locus *Gn1a* (*Grain number 1a*) was the most effective QTL for increasing grain number. This QTL was mapped in a population of 13,000 F<sub>2</sub> plants to a small region of 6.3 kb, which harboured a single predicted ORF corresponding to the *OsCKX2* gene. In control experiments, transgenic rice plants expressing anti-sense or sense *OsCKX2* constructs produced an increased or reduced grain number, respectively, confirming that changes in *OsCKX2* expression are causally related to changes in grain yield (Ashikari *et al.*, 2005). Analysis of *OsCKX2* promoter-*GUS* fusion gene revealed high expression in the vascular tissue of developing culms, inflorescence meristems and young flowers, suggesting that *OsCKX2* controls cytokinin transport to the meristem and thus may regulate meristem size and/or flower number. Accordingly, the *OsCKX2* transcripts were less abundant in the inflorescence meristem of rice varieties with a large grain number (Ashikari *et al.*, 2005). This outstanding work is a convincing demonstration that control of cytokinin metabolism contributes to productivity in rice.

Ectopic expression of *CKX* genes in transgenic plants leads to a strong reduction of endogenous cytokinin levels and development of aberrant phenotypic traits of cytokinin deficiency, which can be indicative of cytokinin-dependent processes *in planta* (Werner *et al.*, 2001; 2003; Yang *et al.*, 2003; Galuszka *et al.*, 2004). From these studies, it was for instance concluded that cytokinin regulates organ growth in opposite ways. The hormone is required to maintain growth and development of the shoot, but is a negative regulator for root growth at physiological concentrations (Werner *et al.*, 2001; 2003). This conclusion was fully confirmed by the analysis of cytokinin-receptor mutants (see below).

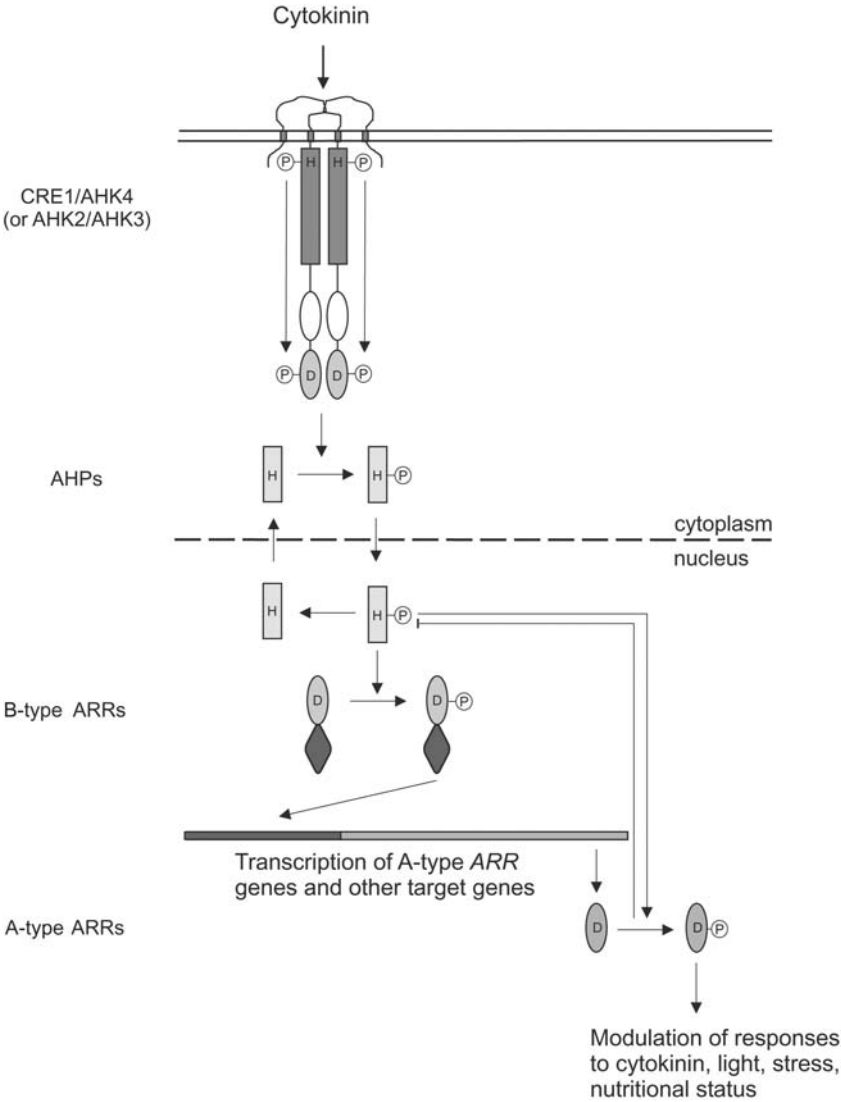
### 4.3 Cytokinin signal transduction

Through numerous experiments it became apparent that the cytokinin signal is perceived in *Arabidopsis* by three membrane-located histidine kinase receptors (AHK2, AHK3, CRE1/AHK4) and transduced via a His-to-Asp phospho-relay system (Hwang & Sheen, 2001; Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Yamada *et al.*, 2001). The general functioning of the cytokinin signaling system as outlined briefly in the following has been addressed in many recent excellent reviews to which the reader may refer for details and earlier work on cytokinin signaling (Urao *et al.*, 2000b; Haberer & Kieber, 2002; Hwang *et al.*, 2002; Oka *et al.*, 2002; Kakimoto, 2003; Heyl & Schmölling, 2003; Grefen & Harter, 2004). In its simplest form, the signaling system consists of two proteins – hence the name two-component system (TCS) – a membrane bound receptor kinase, which senses the signal and autophosphorylates, and a response regulator, which upon phosphorylation by the receptor kinase, activates the transcription of its target genes or initiates another output reaction (West & Stock, 2001). *Arabidopsis* uses a multistep phospho-relay system, which comprises additional signaling steps. After the binding of cytokinin to the extracellular ligand-binding domain, the so-called CHASE domain, a dimer of the receptor histidine kinase (AHK) transphosphorylates itself at a conserved histidine residue. This signal is then transferred within the receptor protein to an aspartate residue in the response regulator domain. Subsequently, the phosphoryl group is transferred to a histidine phospho-transfer protein (AHP), which then translocates to the nucleus, where it activates the B-type response regulators (ARRs) by phosphorylation of a canonical aspartate residue. These response regulators activate the transcription of their target genes, one group of which is the A-type ARRs. These are involved in a feedback loop regulating the activity of cytokinin signaling and link the cytokinin signaling pathway with other cellular signaling pathways (Fig. 4.3) (Heyl & Schmölling, 2003).

As most experiments in cytokinin signal perception and transduction have been done in *Arabidopsis*, this review focuses on new results in this model plant since 2003. However, research in other plant species will be addressed as well. First we will focus on the new discoveries in the field of cytokinin perception and then report the progress made in the area of cytokinin signal transduction by discussing each protein family separately. Starting with phylogeny, we will subsequently address expression pattern, subcellular localization and biochemical studies, followed by results of mutant analysis considering functional aspects.

#### 4.3.1 Cytokinin signal perception

Like other plant histidine kinases the cytokinin receptors consist of an input domain, a histidine kinase domain and a receiver domain. In a phylogenetic tree of the *Arabidopsis* histidine kinase domains, all AHK proteins, AHK2, AHK3 and CRE1/AHK4, cluster together, indicating their close evolutionary relationship (Yonekura-Sakakibara *et al.*, 2004). Interestingly, the *Arabidopsis* cytokinin receptors are more closely related to their respective maize orthologs than to any of the other



**Figure 4.3** A model for cytokinin signal transduction via the two-component system. The structure of CRE1/AHK4 is shown as an example. Ligand binding induces autophosphorylation, the phosphoryl group is then transferred via an His-to-Asp-relay by phosphotransmitter proteins (AHPs) from the cytoplasm to type-B response regulators (ARRs) in the nucleus. Type-B response regulators transcribe target genes, among them type-A *ARR* genes. The model predicts that type-A ARRs down-regulate the primary cytokinin signal response via a negative feedback loop acting on AHPs. In addition, A-type ARRs modulate downstream activities of cytokinins in a positive or negative fashion. D: aspartate residue; H: histidine residue; P: phosphoryl group.

*Arabidopsis* His-kinases, indicating that the founding member of the gene family has evolved before the separation of mono- and dicots. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, *in situ* hybridization and studies of the expression of promoter-*GUS* fusion genes have shown that *CRE1/AHK4* is mainly expressed in the vasculature of the root but also has weak expression in the aerial parts of the plant (Mähönen *et al.*, 2000; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). In contrast, *AHK2* and *AHK3* are expressed in all parts of the plant (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). No data on the subcellular localization of the receptors *in planta* have been published, but it is generally assumed that they are located at the plasma membrane. The current signal transduction model (Fig. 4.3) predicts that the receptors dimerize and autophosphorylate upon ligand binding. This model is derived from the way other His-kinases function and no experimental proof has been provided thus far for cytokinin receptors. In support of further transmission of the signal through AHPs, it was shown that all three AHKs can interact with all five AHPs (Suzuki *et al.*, 2002; Dortay *et al.*, unpublished data).

Biochemical binding assays and response studies performed in a heterologous host showed that the cytokinin receptors differ in their binding specificities with different cytokinins. In *in vitro* binding studies *CRE1/AHK4* bind the bases tZ and iP ( $K_D \sim 4.5$  nM) very well (Yamada *et al.*, 2001). A systematic study compared the ligand specificities of *CRE1/AHK4* and *AHK3* using a bacterial assay and confirmed the preference of both receptors for the bases of isoprenoid-type cytokinins (Spichal *et al.*, 2004). It was also shown that *CRE1/AHK4* has a narrow spectrum of ligands while *AHK3* recognizes a much wider range of cytokinins, albeit with a much lower relative affinity (Spichal *et al.*, 2004). This difference would allow for specification of the different cytokinin signals at the level of the receptors and raises the possibility that the different cytokinins trigger partially specific cellular responses.

Binding specificities were also measured for the different cytokinin receptors of maize, *ZmHK1*, *ZmHK2* and *ZmHK3a* (Yonekura-Sakakibara *et al.*, 2004). It was found that also these three receptors preferentially recognize the free bases of the hormone. Intriguingly, *ZmHK1*, which is most closely related to *CRE1/AHK4*, showed also a stronger preference for some specific cytokinins (iP, iPA). *ZmHK2*, the *AHK3* homologue and *ZmHK3a*, which is most similar to *AHK2*, recognize a much broader spectrum of ligands. All three cytokinin receptors were able to respond to cZ underpinning the relevance of this cytokinin in maize (Yonekura-Sakakibara *et al.*, 2004).

The analysis of single, double and triple receptor mutants of *Arabidopsis* demonstrated that the three receptors have at least a partial overlap in their function. Knockout mutants of single receptor genes showed no, or only limited, cytokinin resistance and no obvious plant phenotypes. But simultaneous mutation of all three receptors caused complete cytokinin resistance and strong plant growth inhibition (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). Triple mutants did not show any cytokinin response, such as inhibition of root elongation or induction of cytokinin response genes. Nevertheless, these plants were able to form roots, stem, leaves and flowers, although this resulted in small and infertile plants (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). These results confirmed

that cytokinins are an important class of plant hormones, but the fact that a plant body was formed in the absence of cytokinin responsiveness raised the question of whether cytokinins are necessary to establish a basic body plan. Several explanations for the triple mutant phenotype are possible. For example, it could be that in the absence of receptors a basic activity level of cytokinin signaling is maintained by other two-component signaling proteins that cross-feed into the cytokinin response system. It could also be that a separate cytokinin signaling circuit exists that ensures basic cytokinin-dependent cellular functions in the absence of the TCS receptor-derived signal.

Further studies of single and double receptor mutants resulted in uncovering the specificity of the different receptors for particular developmental processes. The most prominent example is the *ahk2 ahk3* double mutant, which exhibited a semi-dwarf shoot phenotype, including compact rosette leaves and reduced inflorescence stem length, indicating that cytokinin is a positive regulator of shoot development and that AHK2 and AHK3 functions dominate in the shoot (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). In contrast, the *ahk2 cre1/ahk4* and *ahk3 cre1/ahk4* mutants looked like wild-type plants (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). However, a detailed analysis has revealed distinct functions of certain receptor combinations in various developmental processes, such as root branching, chlorophyll retention during dark-induced leaf senescence and seed germination (Riefler *et al.*, unpublished data).

Comparative analysis of the induction of cytokinin primary response genes in *ahk3*, *cre1/ahk4* single mutants and the *ahk3 cre1/ahk4* double mutant revealed also specificity of the receptor for different early responses and possibly branching of pathways immediately downstream of the receptors (Franco-Zorrilla *et al.*, 2005). In the single mutants the induction of *ARR4* by cytokinin was lowered and it was further lowered, although still detectable, in the double mutant, indicating that either receptor can mediate signal transfer of this gene to some extent, but full induction is only achieved by their combined activities. In contrast, the induction of both *ARR6* and *ARR15* was only slightly and to a similar extent reduced in the single and double mutants, demonstrating that AHK2 is sufficient to mediate almost the full response. The induction of yet another primary response gene, *ARR16*, was abolished in both single mutants and the double mutant, indicating that combined action of AHK3 and CRE1/AHK4 is required for its induction (Franco-Zorrilla *et al.*, 2005).

The investigation of physiological parameters in the receptor mutants has revealed some previously unknown functions of cytokinin in mineral nutrition. Cytokinin was found to be a negative regulator of sulphur acquisition, its function in this process being mainly mediated by CRE1/AHK4 (Maruyama-Nakashita *et al.*, 2004). Cytokinin down-regulates the expression of high-affinity sulphate transporter genes *SULTR1;1* and *SULTR1;2*, which are induced by sulphur limitation and repressed by the presence of reduced sulphur. It was then shown that the down-regulation of *SULTR* genes as well as the reduction of sulphate transport was less strong in a *cre1/ahk4* mutant, indicating that the cytokinin response system is relevant to sulphate acquisition. The authors suggest the existence of at least two independent modes



of regulation for sulphate acquisition, one dependent on sulphate depletion and one dependent on cytokinin (Maruyama-Nakashita *et al.*, 2004).

It became also clear that cytokinin plays a crucial role in the negative regulation of the phosphate (Pi) starvation response. In a mutant screen to identify proteins involved in the cytokinin repression of Pi-starvation-induced genes, Franco-Zorrilla and colleagues found different mutant alleles of *cre1/ahk4*, emphasizing the importance of this receptor in this process (Franco-Zorrilla *et al.*, 2002). However, the *cre1/ahk4* mutations did not result in full impairment of cytokinin repression of Pi-starvation-responsive genes suggesting redundancy. Indeed, later experiments using *ahk3* and *cre1/ahk4* single and double mutants showed that AHK3 is also involved in this process and has an additive effect (Franco-Zorrilla *et al.*, 2005). Split-root experiments suggested that local perception of cytokinin is required to block the Pi-starvation response. In the same paper it was shown that *ahk3 cre1/ahk4* double mutant seedlings are more sensitive to sucrose in the media than wild-type seedlings and that AHK3 and CRE1/AHK4 are relevant to cross-talk between sugar, cytokinin and Pi signaling (Franco-Zorrilla *et al.*, 2005). Together these experiments demonstrate the connection between cytokinin and various physiological processes at the molecular level. More detailed analysis of those interactions will reveal which parts of the cytokinin signaling pathway mediate this cross-talk.

Previous work on the *wol* mutant has shown that cytokinin is crucial for vascular development (Mähönen *et al.*, 2000). *wol* is a specific *cre1/ahk4* mutant allele which carries a single amino acid exchange (T278I) in the CHASE-domain and renders it unable to recognize cytokinin. The *wol* mutant seedlings show strong defects in root metaxylem formation (Mähönen *et al.*, 2000). It is not yet understood why only this class of mutants causes this phenotype, while other *cre1/ahk4* mutants carrying other alleles form an apparently normal vasculature. Analysis of a novel *wol* allele, which is mutated in the His-kinase domain, has revealed another interesting feature of CRE1/AHK4 signaling (Garcia-Ponce de Leon *et al.*, 2004). Importantly, the *trans*-heterozygote between the two different *wol* mutants displayed intragenic complementation for the vascular differentiation process, but were still cytokinin insensitive in root elongation and callus proliferation assays. Thus the canonical CRE1/AHK4 cytokinin signaling response was not restored. The authors conclude that canonical cytokinin signaling and procambial cell proliferation may operate through at least partially separate pathways and speculate that CRE1/AHK4 signals the repression of the proliferation of procambial cells as a monomer. Furthermore, these experiments suggest that a TCS-independent pathway, regulating the developmental program of metaxylem formation, might originate at CRE1/AHK4 (Garcia-Ponce de Leon *et al.*, 2004).

#### 4.3.2 Cytokinin signal transduction

##### 4.3.2.1 Histidine phospho-transfer proteins

Phylogenetic analysis of the five AHPs places AHP2, AHP3 and AHP5 in a subgroup, while AHP1 and AHP4 are somewhat more distantly related (Tanaka *et al.*,



2004). This grouping is also reflected in the expression pattern. Northern blot analysis revealed that *AHP2*, *AHP3* and *AHP5* are expressed in all tissues, *AHP1* mainly in roots and *AHP4* almost exclusively in different shoot tissues. Interestingly, transcripts of different size were found in leaves, stems and flowers, indicating differential splicing and, possibly, organ-specific peptides and functions (Tanaka *et al.*, 2004). The model of cytokinin signal transduction predicts that after phosphorylation the AHPs transfer the signal from the presumed site of perception, the cell membrane, to the location of transcriptional regulation, the nucleus (Hwang & Sheen, 2001) (Fig. 4.3). Subcellular localization studies using GFP-tagged AHPs indicated a predominant localization in the cytoplasm and only a minor proportion in the nucleus, at least under the experimental conditions that were tested (Tanaka *et al.*, 2004). However, in an earlier study in a protoplast system it was shown for some AHPs that they indeed translocate from a mainly cytosolic localization to the nucleus in a cytokinin-dependent manner (Hwang & Sheen, 2001), which is in accordance with their proposed function.

The central role of the AHPs as mediators of the cytokinin signal requires that they can interact with AHKs and the ARRr. Numerous experiments have shown such interactions (for review see Heyl & Schmölling, 2003), and additionally it was shown that AHPs are able to acquire a phosphoryl group from *E. coli* His-kinases, providing additional evidence for the current model of cytokinin signaling (Tanaka *et al.*, 2004). However, only a recent large-scale protein–protein interaction analysis encompassing almost all of the TCS proteins revealed that in addition to the high degree of redundancy, there are also some specific interactions. For example, only one or a subset of the AHPs interacts with some specific ARRr (Dortay *et al.*, unpublished data). This type of specific interaction might allow for generating signal specificity within the otherwise redundant cytokinin signaling pathway as the signals transmitted via the AHPs may regulate not all, but only a subset of the ARRr in some cells. There are no published data available for *AHP* mutants in *Arabidopsis*. However, down-regulation of *CrHPI*, a homologous gene of *Catharanthus roseus*, by an RNAi approach abolished the inducibility of A-type *CrRRs* in a cell culture (Papon *et al.*, 2004). This result provides strong support for a crucial role of histidine-containing phospho-transfer (HPs) in cytokinin signaling.

HP proteins were also studied in wheat and maize, which both have at least three HP proteins, TaHP1–TaHP3 and ZmHP1–ZmHP3 (Asakura *et al.*, 2003; Ma, 2005). Different types of experiments indicate a similar role of HPs in maize as was found in *Arabidopsis*. Yeast two-hybrid assays showed that they physically interact with both cytosolic and nuclear response regulator proteins, suggesting branching of the cytokinin pathway into the different compartments (Asakura *et al.*, 2003). ZmHP2 is the only plant HP of which the crystal structure is known. While the overall structure of the protein is similar to those known from bacteria, some non-conserved residues, which are surrounded by the highly conserved, functional amino acids, might be important for protein–protein interaction (Sugawara *et al.*, 2005).

#### 4.3.2.2 Response regulators

The response regulator proteins are defined by their response regulator domain. This type of protein has been identified in more than 25 different plant species (PFAM database, 2005; <http://www.sanger.ac.uk/Software/Pfam/>). The response regulator protein family can be further subdivided into two major classes, the A- and the B-type RRs. In addition to the response regulator domain, the A-type RRs contain only short N- and C-terminal extensions which do not contain any other known functional protein domains. A-type RRs are induced by cytokinin and thought to be involved in a negative feedback mechanism of the cytokinin signaling and in the modulation of the cellular response to cytokinins (Fig. 4.3) (Hwang & Sheen, 2001; To *et al.*, 2004). However, the molecular functions of the A-type RRs remain to be elucidated.

The B-type RRs have in addition to the response regulator domain a DNA-binding domain, the so-called GARP motif, and some were shown to function as transcriptional activators (Mizuno, 2004). The B-type RR genes are not induced by cytokinin and are involved in the transcription of the cytokinin primary target genes (Hwang & Sheen, 2001).

In addition, there are so-called pseudo RRs (five in *Arabidopsis*). Although they contain a RR domain the conserved aspartate of this domain, which is required for the His-to-Asp phospho-relay, is substituted by a glutamate. Furthermore, some pseudo RRs contain a common signature motif (CCT). Some proteins of this group are implicated in the maintenance of the circadian rhythm (Mizuno, 2004) and it is generally believed that pseudo RR do not participate in cytokinin signaling. However, a genome-wide analysis of cytokinin-regulated genes showed that the expression of some *Arabidopsis* pseudo RR genes is affected by the cytokinin status, indicating that a functional link between cytokinin and this gene family may exist (Brenner *et al.*, 2005).

**4.3.2.2.1 B-type response regulators** Based on the amino acid sequence of the RR domain, the B-type response regulators of *Arabidopsis* can be divided into three subclasses. The biggest subclass consists of two pairs of closely related ARR (ARR1/ARR2 and ARR10/ARR12) and three ARRs, which have no close homologue (ARR11, ARR14, ARR18). The other two subclasses consists of one pair of related protein each, ARR13/ARR21 and ARR19/ARR20 (Mason *et al.*, 2004; Tajima *et al.*, 2004). This division into different subclasses is reflected in the expression patterns. Promoter-*GUS* fusions as well as RT-PCR experiments showed that the members of the first subclass are expressed in almost all tissues, with the exception of *ARR18*, which is flower-specific and *ARR14*, for which transcripts were not found in roots. Members of the other two subclasses are expressed only in reproductive tissues, indicating a role during the reproductive phase of development (Mason *et al.*, 2004; Tajima *et al.*, 2004). An exception is *ARR13*, for which no transcript was detected by RT-PCR and only the promoter-*GUS* fusion yielded some expression of this gene in the leaves (Mason *et al.*, 2004). However, it should be also mentioned that within each subgroup highly localized expression can be detected as well (Table 4.1).

**Table 4.1** Expression characteristics of cytokinin-receptor genes and components of the cytokinin signaling pathway in *Arabidopsis*

Gene name	MIPS designation	Tissue-specific gene expression (RT-PCR or reporter GUS assays)	Gene expression (Genevestigator <sup>1</sup> )
<i>Arabidopsis cytokinin receptors</i>			
<i>AHK2</i>	At5g35750	Leaf veins, petioles, inflorescence stems, floral organs, siliques and moderately in root meristems (Higuchi <i>et al.</i> , 2004; Nishimura <i>et al.</i> , 2004)	Strongest expression in shoot apex, node and radicle, expression in all tissues, except for stamen, present at all growth stages, induced by isoxaben
<i>AHK3</i>	At1g27320	Ubiquitously in roots, leaves, inflorescence stems, flowers (Higuchi <i>et al.</i> , 2004; Nishimura <i>et al.</i> , 2004)	Strongest expression in senescent and cauline leaves, medium expression in all tissues, present at all growth stages, induced by N-starvation, programmed cell death and various abiotic stresses
<i>CRE1/AHK4</i>	At2g01830	High in vascular tissues of the apical part of root meristem, moderate in inflorescence stems and pedicels and low in leaves (Higuchi <i>et al.</i> , 2004; Nishimura <i>et al.</i> , 2004)	Strongest expression in shoot apex, expressed mainly in roots and seedlings, present at all growth stages, strong induction by <i>A. tumefaciens</i> , cytokinin, isoxaben
<i>Arabidopsis histidine phospho-transfer protein gene family (AHP)</i>			
<i>AHP1</i>	At3g21510	Seedlings and roots (Tanaka <i>et al.</i> , 2004)	Strongly expressed in root elongation zone and lateral roots, weaker expression in seedlings, expressed during vegetative phase of the plant, but weak expression also in reproductive stages, strongly induced by various light conditions, programmed cell death and oxidative and osmotic stress, weakly induced by cytokinin
<i>AHP2</i>	At3g29350	Seedlings, roots, leaves, stem, flowers, siliques (Tanaka <i>et al.</i> , 2004)	Strongest expression in cell culture, medium expression in all tissues, present at all growth stages, not induced by chemicals or stress
<i>AHP3</i>	At5g39340	Seedlings, roots, leaves, stem, flowers (Tanaka <i>et al.</i> , 2004)	Strongest expression in the root elongation zone and lateral roots, weak expression in all tissues, present at all growth stages, induced by nematodes, isoxaben, ABA, ethylene, cytokinin, N-starvation, heat and osmotic stress
<i>AHP4</i>	At3g16360	Seedlings, roots, leaves, stem, flowers, siliques (Tanaka <i>et al.</i> , 2004)	Strongest expression in cauline and senescent leaves, very weak to not present in other tissues, present at all growth stages, strongly induced by various light conditions, mild induction by nematodes, mycorrhiza, K-starvation and

(Continued)

**Table 4.1** (Continued)

Gene name	MIPS designation	Tissue-specific gene expression (RT-PCR or reporter GUS assays)	Gene expression (Genevestigator <sup>1</sup> )
			various stress responses, including heat, osmotic, oxidative and salt stress
<i>AHP5</i>	At1g03430	Seedlings, roots, leaves, stem, flowers, siliques (Tanaka <i>et al.</i> , 2004)	Strongest expression in cauline and senescent leaves, medium expression in all tissues and at all growth stages, induced by salicylic acid (SA)
<i>Arabidopsis type B response regulator genes (type B ARR)</i>			
<i>ARR1</i>	At3g16857	Seedlings, root elongation zone, hydathodes, in vascular tissue of shoots, leaves, stems, also developing anthers and siliques (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	na
<i>ARR2</i>	At4g16110	Seedlings, root elongation zone, lateral roots, hydathodes, vascular tissues of shoots and leaves, stem, also developing anthers and siliques (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in sepals and senescent leaves, medium expression in all tissues and at all growth stages, strongly induced by programmed cell death, N-starvation and osmotic stress
<i>ARR10</i>	At4g31920	Seedlings, root elongation zone, lateral roots, hydathodes, vascular tissue of shoots and leaves, stem, also developing anthers and siliques, all meristems (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in the node and the root elongation zone, medium expression in all tissues, expressed at all growth stages, induced by N-starvation, mycorrhiza, nematodes, <i>A. tumefaciens</i> , prohexadione, syringoline
<i>ARR11</i>	At1g67710	Seedlings, hypocotyl, roots, shoots, leaves, stem, flowers, siliques (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in the radicle and in lateral roots, present at early vegetative and late reproductive stages, induced by biotic infections, ABA, brassinolide (BL), ethylene, N-starvation, programmed cell death and various abiotic stresses
<i>ARR12</i>	At2g25180	Seedlings, root elongation zone, lateral roots, hydathodes, in vascular tissue of shoots and leaves, stem, also developing anthers and siliques (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in lateral roots, elongation zone, expression in all tissues, present at all growth stages, induced by <i>A. tumefaciens</i> , nematodes, isoxaben, cytokinin, light and osmotic stress
<i>ARR13</i>	At2g27070	Young leaves, vascular tissue of adult leaves (Mason <i>et al.</i> , 2004)	na
<i>ARR14</i>	At2g01760	Seedlings, shoots, young leaves, stems, flowers, siliques (Mason <i>et al.</i> ,	Strongest expression in carpel and shoot apex, medium expression in

(Continued)

**Table 4.1** (Continued)

Gene name	MIPS designation	Tissue-specific gene expression (RT-PCR or reporter GUS assays)	Gene expression (Genevestigator <sup>1</sup> )
		2004; Tajima <i>et al.</i> , 2004)	all tissues, present at all growth stages, induced by nematodes, isoxaben, light, Zn-ions and heat stress
<i>ARR18</i>	At5g58080	Flowers, siliques, young leaves (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in stamen and juvenile leaves, medium expression in all tissues, present at all growth stages, induced by isoxaben, ethylene, GA, methyl jasmonate, cytokinin, N- and S-starvation
<i>ARR19</i>	At1g49190	Flowers, siliques, trichomes of young leaves (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in siliques, medium expression in all tissues, present at all growth stages, induced by many abiotic and biotic stresses, as well as numerous chemicals
<i>ARR20</i>	At3g62670	Junction of sepals and pedicels, flowers, siliques (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	na
<i>ARR21</i>	At5g07210	Junction of sepals, carpels and pedicels, flowers, immature seeds (Mason <i>et al.</i> , 2004; Tajima <i>et al.</i> , 2004)	Strongest expression in siliques and seeds, weak expression in all tissues, very weak in all other stages, except the end of the reproductive phase, induced by biotic stresses, numerous chemicals and by programmed cell death
<i>Arabidopsis type A response regulator genes (type A ARR)</i>			
<i>ARR3</i>	At1g59940	Vascular tissue in shoots and roots (To <i>et al.</i> , 2004)	Strongest expression in lateral roots and elongation zone of the roots, weak expression in seedlings, present at all growth stages, but strongest in early developmental stages, induced by <i>A. tumefaciens</i> , various chemicals, cytokinin, programmed cell death and different light conditions
<i>ARR4</i>	At1g10470	Vascular tissue in shoots and roots (To <i>et al.</i> , 2004)	Strongest expression in the hypocotyl, medium expression in all tissues but seed and petals, present at all growth stages, induced by <i>A. tumefaciens</i> , isoxaben, norflurazon, cytokinin, numerous light conditions and programmed cell death
<i>ARR5</i>	At3g48100	Shoot and root meristem (To <i>et al.</i> , 2004)	Expression mainly in roots and seedlings, present at all growth stages, induced by <i>A. tumefaciens</i> , cycloheximide, cytokinin,

(Continued)

**Table 4.1** (Continued)

Gene name	MIPS designation	Tissue-specific gene expression (RT-PCR or reporter GUS assays)	Gene expression (Genevestigator <sup>1</sup> )
			programmed cell death, cold, salt, UV-B and wounding stress
<i>ARR6</i>	At5g62920	Shoot meristem (To <i>et al.</i> , 2004), roots (Imamura <i>et al.</i> , 1998)	Strongest expression in root, stem and hypocotyl, medium expression in most tissues, present at all growth stages, induced by <i>A. tumefaciens</i> , norflurazon, cytokinin, different light conditions, programmed cell death and cold stress
<i>ARR7</i>	At1g19050	na	Expression mainly in stems, leaves and seedlings, present at all growth stages, induced by cytokinin
<i>ARR8</i>	At2g41310	Roots and seedling vasculature (To <i>et al.</i> , 2004)	Strongest expression in lateral roots and root elongation zone, weak expression in all tissues, present at all growth stages, induced by <i>A. tumefaciens</i> , cycloheximide, ethylene, GA, cytokinin, many different light condition, programmed cell death
<i>ARR9</i>	At3g57040	Roots and seedling vasculature (To <i>et al.</i> , 2004)	Strongest expression in hypocotyl, stem and in lateral roots and root elongation zone, medium expression in all tissues, present in all tissues growth stages, induced by <i>A. tumefaciens</i> , cytokinin, numerous light conditions and programmed cell death
<i>ARR15</i>	At1g74890	Roots (Kiba <i>et al.</i> , 2002)	Expression mainly in hypocotyl and SAM, present at all growth stages, induced by various biotic stresses and chemicals, cytokinin and cold stress
<i>ARR16</i>	At2g40670	Roots (Kiba <i>et al.</i> , 2002)	Strongest expression in petals, sepals, stamen, cauline and senescent leaves, expressed in later growth stages, induced by <i>A. tumefaciens</i> , <i>P. infestans</i> , different chemicals, ABA, ethylene, cytokinin and programmed cell death
<i>ARR17</i>	At3g56380	na	Expression in all tissues, present in all growth stages, induced by cycloheximide, all phytohormones, but GA and SA, K- and N-starvation
<i>ARR22</i>	At3g04280	Leaves, stem, flowers, siliques (Kiba <i>et al.</i> , 2004)	Expression in siliques and seeds, only expressed in late reproductive

(Continued)

**Table 4.1** (Continued)

Gene name	MIPS designation	Tissue-specific gene expression (RT-PCR or reporter GUS assays)	Gene expression (Genevestigator <sup>1</sup> )
			stage, induced by various biotic stresses and chemicals, ABA, ethylene, nutrient starvation and programmed cell death
<i>ARR24</i>	At5g26594	na	na

na = data not available.

<sup>1</sup><https://www.genevestigator.ethz.ch>, version of 30 August 2005.

All the B-type ARR<sub>s</sub> for which the subcellular localization has been tested (*ARR1*, *ARR2*, *ARR10*–*ARR12* and *ARR19*) were found in the nucleus (Hwang & Sheen, 2001; Lohrmann *et al.*, 2001; Hosoda *et al.*, 2002; Hwang *et al.*, 2002; Mason *et al.*, 2004). This is consistent with their predicted role as transcription factors. In accordance with the model for cytokinin signal transduction (Fig. 4.3), interactions between AHP<sub>s</sub> and B-type ARR<sub>s</sub> have been detected (Imamura *et al.*, 2003; Tanaka *et al.*, 2004).

Mutational analysis has yielded important structural and functional information about B-type ARR<sub>s</sub>. A deletion of the N-terminal RR domain of *ARR1* and *ARR2* led to an increase in transcriptional activity (Sakai *et al.*, 2000), indicating a negative regulatory function of the N-terminal RR domain. The relevance of the N-terminal domain was confirmed for several other B-type RR<sub>s</sub>. Thus, expression of the *ARR2*<sup>D80E</sup> mutant protein, in which the canonical aspartate is substituted by a glutamate, did not rescue the *arr2* mutant, underlining the importance of the His-to-Asp phosphorelay for the function of this protein *in planta*. Around half of wild-type plants overexpressing the *ARR2*<sup>D80E</sup> mutant protein died before flowering. These plants also showed the triple response phenotype even in the absence of ethylene (Hass *et al.*, 2004). However, while the importance of the canonical Asp for the functions of *ARR2* was clearly evident, phosphorylation at this residue was not relevant to DNA-binding of the protein (Hass *et al.*, 2004).

Several studies reporting B-type ARR gene loss-of-function mutants have been published (Sakai *et al.*, 2000; 2001; Horák *et al.*, 2003; Hass *et al.*, 2004). A T-DNA knockout line of *ARR1* did not display any phenotypical alterations, except for longer roots (Sakai *et al.*, 2001). Similarly, a transposon insertion into the *ARR21* gene abolished full-length transcription of the gene, but did not cause any discernible phenotype, supporting the notion of redundant B-type ARR functions (Horák *et al.*, 2003). In contrast to these findings, a detailed analysis of *arr2* knockout mutants showed that cytokinin sensitivity, as well as that of ethylene, were affected in hypocotyl elongation assays. This indicates that this protein may be involved in the signaling of both plant growth regulators (Hass *et al.*, 2004).

Plants overexpressing single B-type ARR<sub>s</sub> were also studied to gain information about their functions *in planta*. These experiments involved the overexpression of



either the whole protein (ARR2) (Hass *et al.*, 2004) or the C-terminal halves of the proteins (ARR11, ARR14, ARR20 and ARR21) (Imamura *et al.*, 2003; Hass *et al.*, 2004; Tajima *et al.*, 2004). The latter approach aimed to relieve the repressor activity of the N-terminal RR domain on the transcription factor function of the C-terminal domains. These truncated transcription factors should function independently of the TCS and constitutively activate their target genes. Overexpression of ARR2 did not cause dramatic phenotypic changes. However, substitution of the conserved Asp80 with Glu created a dominant-active form of ARR2, whose overexpression caused severe pleiotropic growth aberrations, including disturbance in the shoot meristem and an aberrant leaf shape (Hass *et al.*, 2004). Overexpression of a truncated ARR11 protein caused unusual growth of cotyledons, reduced apical dominance, the formation of amorphous carpels and siliques, and enabled callus formation on lower cytokinin concentrations than for the wild type. These changes can be at least partially attributed to altered cytokinin signaling. However, root development was not affected in these plants and A-type RR genes were not up-regulated, which would have been expected if a cytokinin signaling pathway was activated (Imamura *et al.*, 2003). Thus the involvement of ARR11 in cytokinin signaling awaits further confirmation, in particular from loss-of-function experiments. Also overexpression of a truncated ARR14 protein caused a somewhat bushy phenotype (Tajima *et al.*, 2004). Plants overexpressing ARR20 grew normally during the vegetative phase, but showed severe defects in the reproductive organs. The few siliques that were formed were thick and club-shaped, and contained fewer seeds than the wild-type (Tajima *et al.*, 2004). Interestingly, the phenotypic alterations were mainly seen in tissues expressing the wild-type forms of ARR14 and ARR20. This may indicate that either the wild-type protein is required in addition to the mutant form to generate the phenotype or that the tissue is particularly responsive to enhanced expression of the target genes for these transcription factors. This was different for overexpressors of the truncated ARR21, which did not grow past the seedling stage, either developing callus-like cotyledons or starting to bleach after formation of the first few leaves. In these mutant lines a more detailed analysis of A-type RR genes was done, showing that most of them (exceptions were *ARR4*, *ARR7* and *ARR8*) were expressed at higher level than in the wild-type, partly in a stage-specific manner (Tajima *et al.*, 2004). Together the results from overexpression of members of the different B-type ARR subclasses revealed that dominant forms of different B-type RRs activate different developmental pathways. This is shown not only by the different phenotypes of overexpressors, but also by the differential activation of cytokinin response genes. This result is somewhat surprising as the degree of functional redundancy between B-type ARR genes was expected to be very high and indicates that the B-type RR may determine an essential step of signal specification.

It is important to identify the target genes of B-type RRs as these may be responsible for mediating specificity of a given response. Several of their target genes have been identified earlier, including particularly the A-type RR genes (Hwang & Sheen, 2001; Imamura *et al.*, 2003). Numerous additional putative target genes of B-type RRs are among the growing list of cytokinin-regulated genes (Schmülling

*et al.*, 1997; Che *et al.*, 2002; Schäfer & Schmülling, 2002; Hoth *et al.*, 2003; Rashotte *et al.*, 2003; Kiba *et al.*, 2004; Zimmermann *et al.*, 2004; Kiba *et al.*, 2005). A recent genome-wide study lists 71 genes that are significantly up-regulated in *Arabidopsis* seedlings 15 min after cytokinin treatment (Brenner *et al.*, 2005). Among these very early cytokinin response genes were numerous transcriptional regulators, indicating regulation of cytokinin pathways through transcriptional cascades involving proteins unrelated to the TCS protein families (Brenner *et al.*, 2005).

Despite the abundance of cytokinin response genes only few functional analyses of *cis*-regulatory elements have been published so far. Imamura *et al.* (2003) showed that ARR11 recognizes a specific nucleotide sequence, GGATT, while ARR1 and ARR2 bind preferentially to AGATT rather than GGATT (Sakai *et al.*, 2000; Hosoda *et al.*, 2002). Ross and colleagues (2004) identified the promoter of the non-symbiotic haemoglobin-2 (*OsNSHB2*) gene of rice as cytokinin responsive and showed that it can be activated by the *Arabidopsis* ARR1 protein. Promoter studies, deletion analysis and site-directed mutagenesis confirmed the relevance of a predicted ARR1-binding *cis*-regulatory element (Sakai *et al.*, 2000; Hosoda *et al.*, 2002; Oka *et al.*, 2002; Rashotte *et al.*, 2003) for gene regulation. These experiments also indicated that ARR1 requires an additional cytokinin-inducible factor for optimal activation (Ross *et al.*, 2004).

In maize there are three B-type RRs known, ZmRR8, ZmRR9 and ZmRR10 (Asakura *et al.*, 2003). As for the *Arabidopsis* B-type ARRs, they are not inducible by cytokinin and were found to localize to the nucleus. They are also able to interact with the ZmHP proteins, although with differential preference, and are phosphorylated by phospho-ZmHP proteins. Interestingly, analysis of the half-life of these phosphorylated B-type ARRs has revealed that it is about ten-fold longer (~30 min) compared with phosphorylated A-type ZmRRs. This difference could be due to different intrinsic phosphatase activities and may be biologically meaningful regarding the different cellular functions of the proteins (Asakura *et al.*, 2003).

In rice seven B-type *OsRR* genes are predicted from the genomic sequence. One of these, the *Early heading date 1* (*Edh1*), was analysed in greater detail and was shown to be involved in the regulation of the photoperiodic flowering (Doi *et al.*, 2004). The *edh1* mutants have a reduced response to photoperiod and *Edh1* can function as a floral inducer. It could be that this B-type RR is involved in a novel two-component signaling cascade, which is unrelated to cytokinin and probably has no functional equivalent in *Arabidopsis* (Doi *et al.*, 2004).

**4.3.2.2.2 A-type response regulators** Sequence comparison reveals that all A-type ARRs divide into five pairs, which are very closely related to each other (Hutchison & Kieber, 2002; Kiba *et al.*, 2004), indicating a recent duplication event in the genome of *Arabidopsis*. One would expect that closely related genes have similar expression patterns. Indeed, this was found to be the case by promoter-*GUS* fusion experiments by To and colleagues (To *et al.*, 2004). *ARR3* and *ARR4* were found to be expressed in the vascular tissue of shoots and roots, the expression of the

pair *ARR5* and *ARR6* was localized in the meristems of shoots and roots and *ARR8* and *ARR9* expression was mainly in the roots. In all cases the level and the total area of expression increased strongly after treatment with exogenous cytokinin. In RT-PCR experiments for *ARR15* and *ARR16*, which are not closely related, expression was detected in the roots (D'Agostino *et al.*, 2000; Kiba *et al.*, 2002). Again expression of these genes and also of *ARR7* was strongly induced by the addition of cytokinin (D'Agostino *et al.*, 2000; Kiba *et al.*, 2002). For *ARR17* there are currently no expression data available. The kinetics of cytokinin induction differs among A-type ARR. While the transcript accumulation of most genes is induced within 15 min, the accumulation of *ARR8*, *ARR15* and *ARR16* transcripts is delayed and begins more than 30 min after the cytokinin treatment (D'Agostino *et al.*, 2000; Brenner *et al.*, 2005).

Much less is known about the subcellular localization of the A-type ARRs. For *ARR5*, *ARR6*, *ARR7* and *ARR15* a nuclear localization was detected with GFP-fusion proteins, while *ARR16* was found in the cytosol (Hwang *et al.*, 2002; Kiba *et al.*, 2002). *ARR4* was reported to be localized in the nucleus as well as in the cytosol (Hwang *et al.*, 2002). For the other A-type ARRs the localization has not yet been determined.

Several A-type ARRs have been shown to be negative regulators of cytokinin signaling in a protoplast system and their feedback regulation of the signaling pathways has been proposed (Hwang & Sheen, 2001) (Fig. 4.3). Analysis of single and multiple knockout mutants of *ARR3–ARR6*, *ARR8* and *ARR9* has largely confirmed this concept and started to dissect the regulatory functions of these genes in different cytokinin-dependent processes (To *et al.*, 2004). In general, double and higher order *arr* mutants displayed a progressively increasing sensitivity to cytokinin and in all processes analysed the hexuple mutant showed the strongest phenotype. However, in lower order mutants the expressivity of the mutant phenotype depended sometimes on particular mutant combinations. For example, the quadruple mutant *arr3 arr4 arr8 arr9* displayed a much higher sensitivity to cytokinin in the inhibition of lateral root formation than any other quadruple mutant combination tested, indicating their relative importance in regulating this process (To *et al.*, 2004). Consistent with a function as negative regulator of cytokinin signaling is a decreased cytokinin sensitivity of *ARR15*-overexpressing plants (Kiba *et al.*, 2003).

While there is good evidence for the negative feedback regulation of cytokinin signaling by A-type ARRs, there are also examples illustrating that some A-type ARRs might be positive regulators and thus enhance the cytokinin response. Overexpressors of *ARR4* showed an increase in the transcript level of cytokinin response genes and enhanced callus and shoot formation in the presence of cytokinin. In contrast, overexpression of *ARR8* repressed callus and shoot formation, indicating an opposite function (Osakabe *et al.*, 2002). Another piece of evidence for opposite effects of A-type ARRs comes from the work on multiple A-type *arr* mutants. The *arr5* knockout mutant has an altered rosette morphology, which disappeared in the *arr5 arr6* double mutant – indicating that *ARR5* and *ARR6* have opposite functions (To *et al.*, 2004).

How the feedback regulation of the cytokinin signaling pathway by A-type ARR proteins is achieved remains unclear. As many biological functions are mediated via protein–protein interaction, one way to approach elucidation of the mode of action is to find interacting partners – “guilty by association”. For all A-type ARRs tested, interactions with some or all of the AHPs were detected (Suzuki *et al.*, 1998; Imamura *et al.*, 1999; Urao *et al.*, 2000a). However, no direct interaction between the A- and the B-type ARRs was found (Dortay *et al.*, unpublished). This suggests that a negative regulation of the signaling pathway by A-type ARRs operates via their interaction with AHPs, for example by competing with B-type ARRs for the phosphoryl residue. In this scenario, a preferred transfer of the phosphoryl residue to A-type ARRs would prevent activation of B-type ARRs (Fig. 4.3). Additionally, A-type ARRs could possess a phosphatase activity and dephosphorylate the AHPs.

In addition to their interaction with AHPs, A-type ARRs interact with various *Arabidopsis* proteins and may link cytokinin signaling with other cellular signaling pathways (Dortay *et al.*, unpublished results). For example, ARR4 was found to interact with two DNA-binding proteins (Yamada *et al.*, 1998). ARR4 was also shown to interact and thus stabilize the Pfr form of phytochrome B, indicating a positive role in red-light signaling (Sweere *et al.*, 2001). A role for A-type ARRs in light signaling was supported by studies of A-type *arr* mutants, although their increased red light sensitivity suggested a negative regulatory function (To *et al.*, 2004). Neither of these studies determined whether or not A-type ARR protein function was in this case dependent on cytokinin signaling.

A special subgroup of RR proteins was recently described (Kiba *et al.*, 2004). ARR22 and ARR24, which lack the B-type characteristic DNA-binding domain and thus are phylogenetically more closely related to the A-type ARRs are not induced by cytokinin – a hallmark trait of the A-type ARRs. RT-PCR experiments showed that ARR22 is expressed mainly in the reproductive organs of the plant, while weak expression was detected in stems and adult leaves. Expression of ARR24 has not yet been detected. GFP-fusion experiments localized ARR22 mainly to the cytosol (Kiba *et al.*, 2004). In phospho-relay experiments, ARR22 was dephosphorylated in the presence of AHP5 indicating an interaction between these two proteins. Surprisingly, overexpression of ARR22 caused a phenotype similar to the *wol* allele of *CRE1/AHK4*, that is, a severe defect of the vascular tissue in the primary root (lack of phloem formation) and a reduced shoot growth. However, ARR22 overexpressors did not flower, which is dissimilar to the *wol* mutant. Expression analysis using microarrays indicated that ARR22 overexpression attenuated the cytokinin response globally. Overexpression of ARR22 lacking the canonical Asp-residue did not have any effect, suggesting that interference with the phospho-relay of the TCS is a likely cause for the phenotypic changes (Kiba *et al.*, 2004). Collectively the data suggest that ARR22 can act as a particularly effective negative regulator of cytokinin signaling, possibly by competing with other ARRs for the phosphoryl residue of the AHPs. However, the *in planta* function of ARR22 is unclear and whether or not it truly has a role in cytokinin signaling cannot be evaluated on the basis of these gain-of-function studies.

A-type *RR* genes were also identified in maize, rice and *Catharanthus roseus* (Papon *et al.*, 2004; To *et al.*, 2004). In contrast to *Arabidopsis*, the known A-type ZmRRs seem not to be organized in pairs. For *ZmRR1* and *ZmRR4–ZmRR7*, a clear inducibility by cytokinin was shown (Asakura *et al.*, 2003). *ZmRR3* is not induced by cytokinin in the leaves, but its area of expression in the shoot meristem increases after cytokinin treatment. The mRNA of this particular *ZmRR* was detected in the reproductive organs (ear and silk), as well as in the shoot apex and the embryo (Asakura *et al.*, 2003; Giulini *et al.*, 2004). Using GFP fusions *ZmRR1* and *ZmRR2* were shown to be localized mainly in the cytosol, while the other A-type ZmRRs were found exclusively in the nucleus (Asakura *et al.*, 2003). Interestingly, after the phospho-transfer from ZmHPs to A-type ZmRRs, the phosphorylation is rather unstable compared to B-type ZmRRs, which could facilitate fine-tuning of cytokinin signaling (Asakura *et al.*, 2003). A single mutation of a *ZmRR* gene is known, which was caused by a transposon insertion in *ZmRR3* in the *abphyll1* (*abph1*) mutant. The *abph1* is a phyllotaxy mutant which initiates leaves in a decussate pattern, leading to the formation of opposite pairs of leaves instead of single alternating leaves (Giulini *et al.*, 2004). The *ABPH1* (*ZmRR3*) gene is expressed in the SAM and cytokinin causes an enhanced expression and a larger expression domain. This and the fact that both cytokinin treatment and the *abph1* mutation cause an increase in meristem size, led to the hypothesis that ABPH1 controls the phyllotaxy patterning process by negatively regulating the effect of cytokinin on the SAM expansion and thus limiting the space available for leaf primodium (Giulini *et al.*, 2004). This result confirms the previously established role of cytokinin in determining meristem size (Werner *et al.*, 2003) and establishes its role in phyllotaxy.

#### 4.4 Conclusions

In the last 5 years a tremendous amount of knowledge about cytokinin metabolism and signaling has been accumulated. The studies have underpinned the complexity of cytokinin metabolism and provided evidence that the control of the local cytokinin concentration is as relevant as downstream cytokinin signaling processes for the fine-tuning of plant growth and development. One important outcome is that the results clearly necessitate the modification of textbook paragraphs describing cytokinin as solely root-derived hormone. There is accumulating evidence that two different type of cytokinin hormonal activities exist in the plant. On the one hand, a local (paracrine or autocrine) activity, which may regulate mainly cell division and sink strength, and secondly an activity based on long-distance signaling (endocrine-like), in which cytokinin may serve primarily as a root-to-shoot signal and may regulate physiological – for example nitrogen-dependent – processes. Future goals are to find out which are the individual functions of the cytokinin metabolic enzymes and to identify the links that co-ordinate metabolism and signaling. The basic flow of information within the signaling system seems to be understood but many questions about the specific molecular function of the different TCS proteins

remain to be answered. An important aspect of future work is to determine how signal specification is generated in this partially redundant signaling system. Almost certainly protein–protein interaction and gene regulation will play key roles. Furthermore, future research in this area will enable the integration of this signaling system into other pathways within the plant cell, thus to understand the molecular mechanisms that drive the developmental and physiological processes related to cytokinin.

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## 5 Ethylene biosynthesis and signaling: a puzzle yet to be completed

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### 5.1 Introduction

The two-carbon olefin ethylene has fascinated plant biologists for more than a century by its myriad of effects on plant growth and development. However, the earliest record of human manipulation of fruit ripening, related to ethylene, goes back to the 8th century B.C., with the prophet Amos describing himself as a piercer of sycamore fig fruits. Five centuries later, Theophrastus, a Greek philosopher, recognized that the fruits would not ripen unless they were first scraped with an iron claw (Blanpied, 1985). This remarkable effect is now known to be associated to the formation of wound ethylene, which subsequently stimulates ripening. The Russian physiologist Dimitry Neljubow was the first to discover that ethylene is a biologically active compound in plants (Neljubow, 1901). He demonstrated that ethylene was the component of illuminating gas, which caused diageotropic (horizontal) growth of pea seedlings, inhibition of elongation, and swelling. Others confirmed and expanded Neljubow's observations on the so-called "triple response" (Knight *et al.*, 1910). The possibility that ethylene was an endogenous growth regulator and its use to manipulate crop growth and development was investigated in the 1920s and 1930s. In 1934, conclusive evidence that ethylene was a natural product from plants, was presented by the English scientist Gane (1934). A full account on the historical background of the discovery of ethylene as a plant hormone is given by Abeles *et al.* (1992).

The subsequent biochemical dissection of the ethylene biosynthesis pathway further strengthened its acceptance as a true plant hormone, despite the fact that strictly speaking, a gaseous hormone is not translocated and hence does not comply with the original definition of a hormone.

The chemical properties of ethylene are important in understanding its biology (Abeles *et al.*, 1992). The diffusion coefficient of ethylene in air is approximately 10,000 times that in water. Furthermore, ethylene is about 14 times more soluble in lipids than in water. Like other olefins, ethylene binds to metals through a co-ordination bond. Among the metals reported to bind to ethylene are Cu(I) and Ag(I). Copper ions serve as a critical cofactor in high-affinity ethylene binding to the receptors. Application of silver ions can displace copper ions from the receptor proteins and, consequently, block ethylene perception.

Physiological effects of ethylene are detectable at ambient levels as low as 0.1  $\mu\text{l/l}$ . Ethylene is particularly known as a ripening hormone. Yet, it has profound

effects on plants at most developmental stages. The effects range from stimulation of germination, triple response in dark-grown dicotyledonous seedlings, impairment (in most species) or stimulation (in aquatic and semi-aquatic species) of cell expansion, over-induction of flowering in bromeliads, to leaf and petal senescence, and abscission. Furthermore, the production of ethylene is tightly regulated by internal signals during development and in response to environmental stimuli, both biotic and abiotic, including pathogen attack, wounding, hypoxia, ozone, chilling, or freezing.

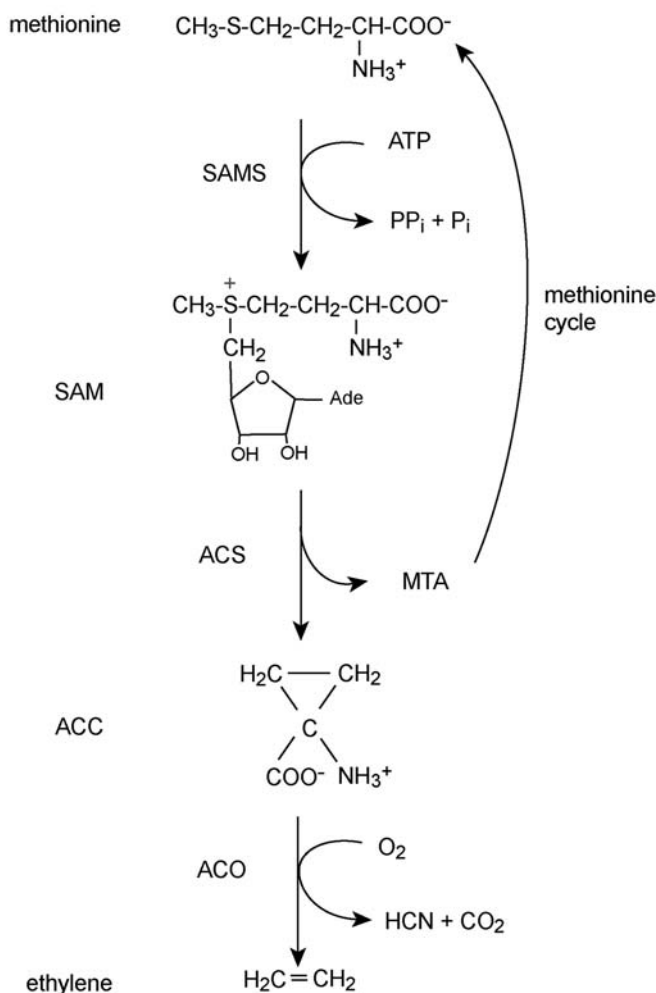
However, the most significant advances in the functional analysis of ethylene in plant physiology have occurred in the past 15 years with thorough studies of the model plant *Arabidopsis*. Exploitation of the most conspicuous effect of ethylene in *Arabidopsis*, the induction of the triple response in the dark characterized by an exaggerated apical hook, a short and thick hypocotyl, and a short root led to the identification of mutants with defective ethylene signaling. The characterization of these mutants enabled cloning and functional analysis of nearly all components in the chain of ethylene biosynthesis and signaling.

The intent of this review is to briefly highlight major findings in ethylene biology and further focus on more recent discoveries that shaped our current view on ethylene biosynthesis and signaling.

## 5.2 Ethylene biosynthesis

In the late 1970s, Yang and co-workers elucidated the ethylene biosynthesis pathway in which methionine is converted to *S*-adenosyl-methionine (*S*-Adomet) by *S*-Adomet synthetase (SAM synthetase) (Fig. 5.1a) (Yang & Hoffman, 1984). *S*-Adomet is the major methyl donor in plants and is involved in a number of biochemical pathways, including polyamine and ethylene biosynthesis (Ravanel *et al.*, 1998). However, only a minor portion of cellular *S*-Adomet is used for 1-aminocyclopropane-1-carboxylic acid (ACC) production; hence, the levels of *S*-Adomet do not limit ethylene production. *S*-Adomet synthetase action is followed by two regulated steps. ACC synthase (ACS), which converts *S*-Adomet to ACC (Adams & Yang, 1979), is the first rate-limiting step in ethylene biosynthesis. In addition to ACC, it also produces 5'-methylthioadenosine (MTA), which is subsequently recycled to methionine. This pathway, known as the Yang cycle (Adams & Yang, 1979), preserves the methyl group for another round of ethylene production. The second point of regulation involves the oxidation of ACC by ACC oxidase (ACO) to form ethylene, CO<sub>2</sub>, and cyanide. The latter is detoxified to  $\beta$ -cyanoalanine by  $\beta$ -cyanoalanine synthase to prevent toxicity of accumulated cyanide during high rates of ethylene biosynthesis. Alternatively, ACC can be conjugated by either malonylation (MACC) or  $\gamma$ -L-glutamylolation (GACC) (Hoffman *et al.*, 1982; Martin *et al.*, 1995), which is regarded as a way to exclude ACC from the ethylene biosynthesis pathway. However, there are some indications that conjugation is not irreversible and under certain conditions MACC may serve as a pool for ACC (Jiao *et al.*, 1986).



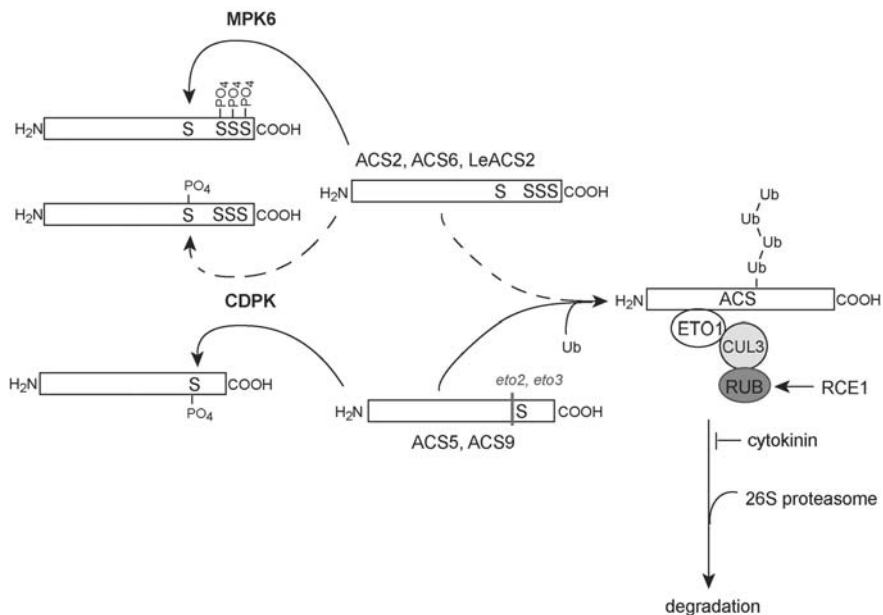


**Figure 5.1a** Overview of ethylene biosynthesis. (a) Methionine is converted to *S*-AdoMet by SAMS. SAM is subsequently used by ACS to yield ACC and MTA. MTA is recycled to methionine in the methionine cycle. ACO oxidizes ACC, resulting in ethylene production. The last step also releases hydrogen cyanide (HCN) and CO<sub>2</sub> as by-products.

We will limit further discussion to the two committed steps in ethylene biosynthesis: ACS and ACO.

### 5.2.1 ACC synthase

In all studied plant species, ACSs are encoded by a multigene family, with high divergence. Biochemical characterization of ACS purified from zucchini (Sato & Theologis,



**Figure 5.1b** Model for regulation of ACS enzyme stability. Phosphorylation on C-terminally located serine residues by either CDPK or MPK6 leads to increased stability. When dephosphorylated, this serine-rich region is necessary for stimulation of ubiquitination and eventual protein degradation. Mutations in that region, like *eto2* or *eto3*, increase protein stability. ETO1 serves as an adaptor for ACS to associate with Cullin3, which is part of an E3 ubiquitin ligase. The activity of this ligase is regulated by rubylation and derubylation through RCE1. Cytokinin inhibits degradation of ACSs by the 26S proteasome.

1989), tomato (Bleecker *et al.*, 1986; Van Der Straeten *et al.*, 1989; 1990), and winter squash (Nakajima & Imaseki, 1986; Nakajima *et al.*, 1990) led to the cloning of the first ACS genes. Due to the release of these sequences a wealth of knowledge on ACS genes was developed shortly thereafter (for an overview, see Fluhr & Mattoo, 1996), including that of the ACS family of both dicot and monocot model systems, *Arabidopsis* (Liang *et al.*, 1992; Van Der Straeten *et al.*, 1992; Rodrigues-Pousada *et al.*, 1993; Abel *et al.*, 1995; Botella *et al.*, 1995; Cary *et al.*, 1995; Vahala *et al.*, 1998; Woeste *et al.*, 1999; Yamagami *et al.*, 2003; Vandenbussche *et al.*, 2003a; Tsuchisaka & Theologis, 2004a & b) and rice (Zarembinski & Theologis, 1993; 1997; Van Der Straeten *et al.*, 1997; 2001; Zhou *et al.*, 2002). With the sequencing of both genomes, the number of identified ACS genes has substantially increased, and in expression studies using RNA gel blot hybridization, cross-hybridization may have occurred due to extremely high homology within certain clades (e.g. *At-ACS4* and *At-ACS8* have over 80% nucleotide identity). The structure of ACSs resembles that of the subgroup I family of pyridoxal 5'-phosphate (PLP)-dependent amino-transferases (Mehta *et al.*, 1993; Capitani *et al.*, 1999). In *Arabidopsis*, the ACS

gene family has 12 members, two of which are functional only as aminotransferases (*ACS10* and *ACS12*) and two others that encode ACS proteins without a proven enzymatic activity (*ACS1* and *ACS3*) (Yamagami *et al.*, 2003). *In vitro* activity assays indicate large differences in enzymatic efficiency among the isoforms, with  $K_m$  values for AdoMet ranging from 8 to 45  $\mu\text{M}$  and  $k_{\text{cat}}$  values varying from 0.19 to 4.82 per s and per monomer. Moreover, the proteins can heterodimerize, suggesting a vast array of complexes with different enzyme activities, which may add to the fine-tuning of ethylene biosynthesis (Tsuchisaka & Theologis, 2004a). The fact that the rate of ethylene biosynthesis is tissue specific and is influenced by environmental factors, implies the presence of a tight regulatory mechanism.

The expression of ACS genes is specifically regulated in various tissues and modulated by environmental and developmental factors. The overlapping patterns of expression of different ACS family members may reflect a combinatorial code for functional heterodimers (Tsuchisaka & Theologis, 2004b). The *ACS8* gene is particularly strongly regulated at the transcript level and its activity is controlled by auxins, light intensity, circadian rhythms, and by negative feedback by ethylene (Yamagami *et al.*, 2003; Vandenbussche *et al.*, 2003a; Thain *et al.*, 2004). *ACS4* and *ACS7* expression are also highly influenced by endogenous factors, including auxin, and external factors (Abel *et al.*, 1995; Wang *et al.*, 2005).

Recently it was demonstrated that ACS enzyme activity is also regulated by protein stability (Fig. 5.1b). Attenuation of this process results in mutant phenotypes. All characterized mutations that specifically lead to ethylene over-production in *Arabidopsis* (*eto* mutants) are the result of stabilization of ACSs (Chae *et al.*, 2003; Wang *et al.*, 2004). The *eto2* mutant produces an ACS5 protein with a modified C-terminus, which renders it hyperstable (Chae *et al.*, 2003). A mis-sense mutation in the C-terminal region of the closely related *ACS9* gene leads to the ethylene over-production in *eto3*, suggesting that both mutations confer disruption of a similar post-translational control. Mutations in the *ETO1* gene also result in ethylene over-production. Wang *et al.* (2004) showed that *ETO1* encodes a protein that directly interacts with ACS5/ETO2. Being a BTB (Broad complex–Tramtrack–Bric-à-brac) domain-containing protein, ETO1 also interacts with CUL3 (cullin3), a subunit of E3 ubiquitin-ligase complexes. Thus, ETO1 links ubiquitin-mediated protein degradation to ethylene biosynthesis and could serve as an adaptor for and a positive regulator of ACS5 breakdown (Wang *et al.*, 2004). The mutated form of ACS5 in *eto2* does not contain the binding site for ETO1 and, as a consequence, it is not recognized for ubiquitin-mediated protein breakdown. Further evidence that ACS5 is post-transcriptionally regulated came from the analysis of the *cin* mutants (*cytokinin insensitive*; Vogel *et al.*, 1998a). Low doses of cytokinin (0.5–10  $\mu\text{M}$ ) stimulate ethylene production in etiolated seedlings of *Arabidopsis*, inducing morphological changes resembling the triple response upon ethylene treatment in darkness (Cary *et al.*, 1995). This was exploited to identify mutants that fail to increase ethylene in response to cytokinin (*cin* mutants; Vogel *et al.*, 1998a). *CIN5* was found to correspond to ACS5 (Vogel *et al.*, 1998b). However, since cytokinin-mediated ethylene production does not correlate with an accumulation of ACS5 mRNA, it can be

concluded that cytokinin increases ACS5 function primarily by a post-transcriptional mechanism. It was demonstrated that *eto2* does not alter the specific activity of the enzyme, but increases the half-life of the ACS5 protein (Chae *et al.*, 2003). In addition, cytokinin treatment increased the stability of ACS5 in the *eto2* background, indicating a mechanism that is at least partially independent of the *eto2* mutation.

Another clade of the ACS family consists of the closely related ACS2 and ACS6 proteins. Like ACS5 and ACS9, these enzymes share a common regulatory control mechanism, being phosphorylated by mitogen-activated protein kinase 6 (MAPK6) on three C-terminal serine residues that are not present in other active ACSs of *Arabidopsis*. Moreover, this phosphorylation increased the stability of the ACS6 protein (Liu & Zhang, 2004). A related tomato isoform, Le-ACS2, can be phosphorylated on yet another serine residue in its C-terminal region by a calcium-dependent protein kinase (CDPK) (Tatsuki & Mori, 2001). Interestingly, a maize CDPK could phosphorylate the corresponding Ser residue of a C-terminal peptide of the *Arabidopsis* ACS5 (Hernandez Sebastià *et al.*, 2004). Thus, depending on the species or protein, phosphorylation is brought about by different mechanisms and is probably the basis for stimulation of protein stability. The four *Arabidopsis* ACS isoforms that share a highly conserved C-terminal sequence (ACS4, ACS5, ACS8, ACS9) may therefore be regulated by CDPK in a similar manner (Chae & Kieber, 2005).

### 5.2.2 ACC oxidase

Biochemical characterization of ACOs progressed well only after cloning of the first gene. Hamilton *et al.* (1991) over-expressed antisense pTOM13 in tomato, a gene with similarity to flavanone-3-hydroxylases, resulting in much reduced ethylene levels. The ACO enzyme requires iron, ascorbate, and CO<sub>2</sub> for its activity (Ververidis & John, 1991). In *Arabidopsis*, like ACSs, ACOs are encoded by a multigene family, albeit with limited divergence. On the basis of sequence similarity, the family has at least 17 members in *Arabidopsis*. Enzyme assays for each of these genes have not been performed yet, making it difficult to speculate on the specific roles of each one in the plant. However, it is generally accepted that in ethylene biosynthesis, ACC oxidation is most often not the rate-limiting step, since the activity is present in most vegetative tissues. However, it has been suggested recently that ACO activity can regulate ethylene production in seedlings (Cancel & Larsen, 2002). This activity is dependent on the intactness of RCE1 (RUB CONJUGATING ENZYME), also linking ACO activity to protein stability. In contrast to vegetative tissues, ACOs are highly regulated during fruit ripening, leaf senescence, and upon wounding and ethylene treatment (Tang *et al.*, 1994; Barry *et al.*, 1996).

Few reports have been made on the expression of ACO genes in *Arabidopsis* (Gomez-Lim, 1993; Raz & Ecker, 1999; Vandenbussche *et al.*, 2003a). ACO2 has been studied in most detail, and being ethylene induced in a feed-forward mechanism, the gene serves as a positive control for ethylene responses (Raz & Ecker, 1999; De Paepe *et al.*, 2004). Microarray studies have revealed that more ACOs are subject to (auto)regulation at the transcript level (Van Zhong & Burns, 2003;

De Paepe *et al.*, 2004). Expression of three ACOs and one ACO-like gene was modified by ethylene (De Paepe *et al.*, 2004). The differential expression of multiple ACOs supports a complex auto-regulatory mechanism in ethylene biosynthesis.

### 5.3 Ethylene signal transduction

Apart from the discovery of *eto* mutants, the classical triple response screening has led to the isolation of various mutants with defects in genes involved in ethylene signaling, including *ethylene resistant* (*etr*), ACC and *ethylene insensitive* (*ain/ein*), and *constitutive triple response* (*ctr*) mutants (Bleecker *et al.*, 1988; Kieber *et al.*, 1993; Van Der Straeten *et al.*, 1993; Roman & Ecker, 1995). Subsequently, the triple response screening was refined towards identification of mutants that display an enhanced ethylene response at low concentrations of the hormone. Using this screen, the *enhanced-ethylene-response 1* (*eer1*) mutant was isolated (Larsen & Chang, 2001). Furthermore, seven other components (*weil-weil7*) have been identified by a low-dose screen for weak ethylene-insensitive mutants (Alonso *et al.*, 2003a; Stepanova *et al.*, 2005). In parallel, other screening methods were applied. One method used responsiveness to an antagonist of ethylene (a compound that interacts with the receptor but acts as an inhibitor of ethylene responses). The *responsive to antagonist* (*ran*) mutant was isolated using *trans*-cyclooctene (TCO) (Hirayama *et al.*, 1999). A second method exploited the phenotype of ethylene/ACC-treated light-grown *Arabidopsis* seedlings, which display an elongated hypocotyl in the presence of ethylene on a low nutrient medium (LNM), a response that is absent in the *etr/ein* mutants and constitutively present in the *ctr1* mutant (Smalle *et al.*, 1997). Using this response, *alh1* (*ACC-related long hypocotyl*) (Vandenbussche *et al.*, 2003b), *eer2* (De Paepe *et al.*, 2005), and *slo1* (*slow*) (Zhang & Van Der Straeten, unpublished results) were isolated. Identification of most of the genes affected in these mutants has enabled modelling of a quite complete ethylene-signaling chain.

The first ethylene receptor gene to be cloned and characterized was from *Arabidopsis* (Chang *et al.*, 1993). Subsequently, putative ethylene receptor orthologs have been discovered in many plant species, including *Rumex* (Vriezen *et al.*, 1997), melon (Sato-Nara *et al.*, 1999), tomato (Tieman *et al.*, 2000), carnation (Shibuya *et al.*, 2002), peach (Bassett *et al.*, 2002), and rice (Goff *et al.*, 2002; Yu *et al.*, 2002). In *Arabidopsis*, ethylene is perceived in the endoplasmic reticulum (ER) membrane by a five-membered family of receptors, named ETR1, ETR2, ERS1, ERS2, and EIN4. The receptor proteins have similarities to two-component regulators of bacteria and yeast. These proteins typically consist of a sensor, with a transmembrane input domain and a transmitter domain with histidine kinase (HK) homology, and a response regulator. The latter contains a receiver domain with a conserved Asp residue, receiving a phosphate from the sensor and further acting by phosphotransfer (a phosphorelay mechanism) to an output domain (usually with transcription factor activity). Three of the ethylene receptor proteins (ETR1, ETR2, and EIN4) have a receiver domain covalently attached at the carboxyl terminal of

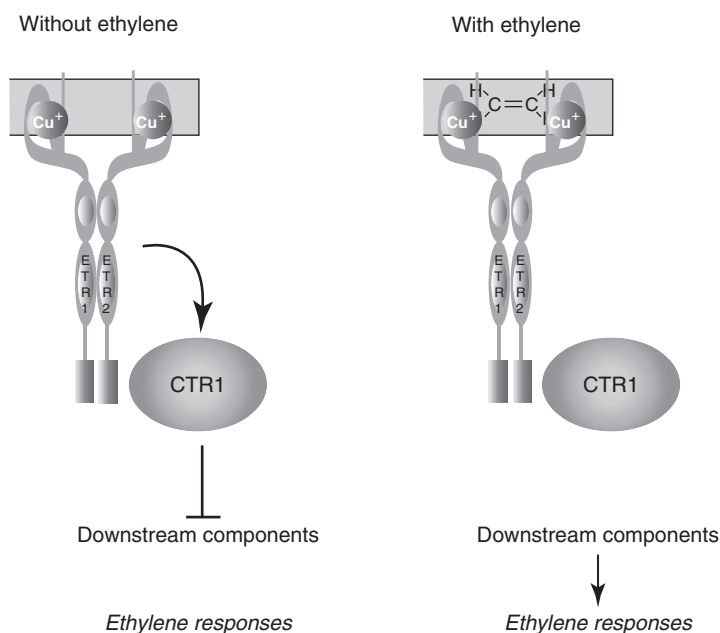
the sensor, and are therefore considered to be hybrid protein kinases (HPKs). In contrast, ERS1 and ERS2 lack a receiver domain and may thus function in conjunction with a response regulator. With respect to their predicted enzymatic function, only ETR1 and ERS1 have the conserved residues typical for HK. This characteristic groups them in subfamily I; while ETR2, EIN4, and ERS2 lack these residues and form subfamily II. Nevertheless, only ETR1 has been shown to possess HK activity (Gamble *et al.*, 1998; 2002). The other receptors have Ser/Thr kinase activity (Klee, 2004). A role for HK activity in ethylene signaling has been questioned, since an ETR1 without HK activity could rescue the *etr1 ers1* loss-of-function (subfamily I) double mutant (Wang *et al.*, 2003; Moussatche & Klee, 2004). This leads to the conclusion that canonical HK activity is probably not required for ethylene receptor signaling. A possible role for the HK activity in ETR1 was proposed by Hass *et al.* (2004) and Qu and Schaller (2004). The *Arabidopsis* response regulator 2 (ARR2) was identified as a signaling component functioning downstream of ETR1 in ethylene signaling (Hass *et al.*, 2004). It was shown that an ETR1-initiated phosphorelay regulates the transcription factor activity of ARR2. This mechanism could constitute a novel signal transfer route from the ER-associated ETR1 to the nucleus for the regulation of ethylene-responsive genes. In conclusion, ETR1 may have a dual functional role in the initiation of ethylene-signal transduction: (1) over a CTR1-dependent pathway (negatively regulated), (2) over an ARR2-dependent pathway (positively regulated). Retention of the HK activity in subfamily I receptors may also provide fine-tuning of the signaling pathway. Recent work indicates that the His kinase domain of ETR1 plays a role in repression of ethylene responses (Qu & Schaller, 2004). Moreover, Binder *et al.* (2004) demonstrated that receiver domains play a role in the recovery from growth inhibition since the *ers1-2 ers2-3* loss-of-function double mutation had no effect on recovery rate after ethylene was removed, while loss-of-function mutations in *ETR1*, *ETR2*, and *EIN4* significantly prolonged the time for recovery.

Analogous to the assay applied by Neljubow (1901), who identified ethylene by making use of its affinity to copper, the ethylene receptors use copper ions as a cofactor for binding ethylene. The copper ions in ETR1 are co-ordinated by two amino acid residues, Cys65 and His 69 (Rodriguez *et al.*, 1999). The copper transporting P-type ATPase, RAN1, is responsible for delivery of copper to the receptor molecules. The ethylene receptors function as homo- and/or heterodimers that are connected by a disulphide bond in the amino terminal region (Schaller *et al.*, 1995). Studies using heterologous expression of the ethylene receptors in yeast showed that ethylene binds to the receptors with a dissociation constant of 0.04  $\mu\text{M}$  with a binding half-life of 12 h. These values match with the rates observed in ethylene-binding/response assays in plants (Chen & Bleecker, 1995).

The receptor mutations first isolated on the basis of conferred ethylene insensitivity are all dominant gain-of-function alleles that introduce a novel or persistent function to the receptor protein. Single loss-of-function receptor mutants do not display a phenotype. Interestingly, of all receptor double mutants produced, only the *etr1 ers1* combination showed a phenotype. The latter mutant and any triple or

quadruple loss-of-function mutants displayed constitutive ethylene responses (Hua & Meyerowitz, 1998; Zhao *et al.*, 2002; Hall & Bleecker, 2003). It was deduced that the ethylene receptors are negative regulators of the ethylene response in the absence of ethylene (Fig. 5.2). Conversely, ethylene must be a negative regulator of receptor function, implying that when ethylene is bound, the receptors are inactivated. This means that the pathway is de-repressed by the hormone, thus leading to ethylene responses.

A mutant that lacks the capability of ethylene binding (as is the case for some of the dominant-insensitive mutations) remains constitutively active and its kinase activity represses ethylene responses. In addition, the model is consistent with higher ethylene sensitivity in the triple and quadruple loss-of-function mutants because less ethylene is necessary to deactivate the remaining functional receptors. Considering that there is differential expression of the receptors, further modulation of ethylene signals may be achieved by altering the relative abundance of each receptor, even though the expression patterns overlap. The differential regulation of the receptor gene family may provide a mechanism to achieve differential sensitivities



**Figure 5.2** Model for function of the ethylene receptors. When ethylene is not present, the receptors suppress ethylene responses by activating CTR1, which inactivates downstream components. Once ethylene binds, the activation of CTR1 is annihilated and the downstream components lead to activation of ethylene response genes, ultimately leading to the phenotypic characteristics associated with ethylene exposure.

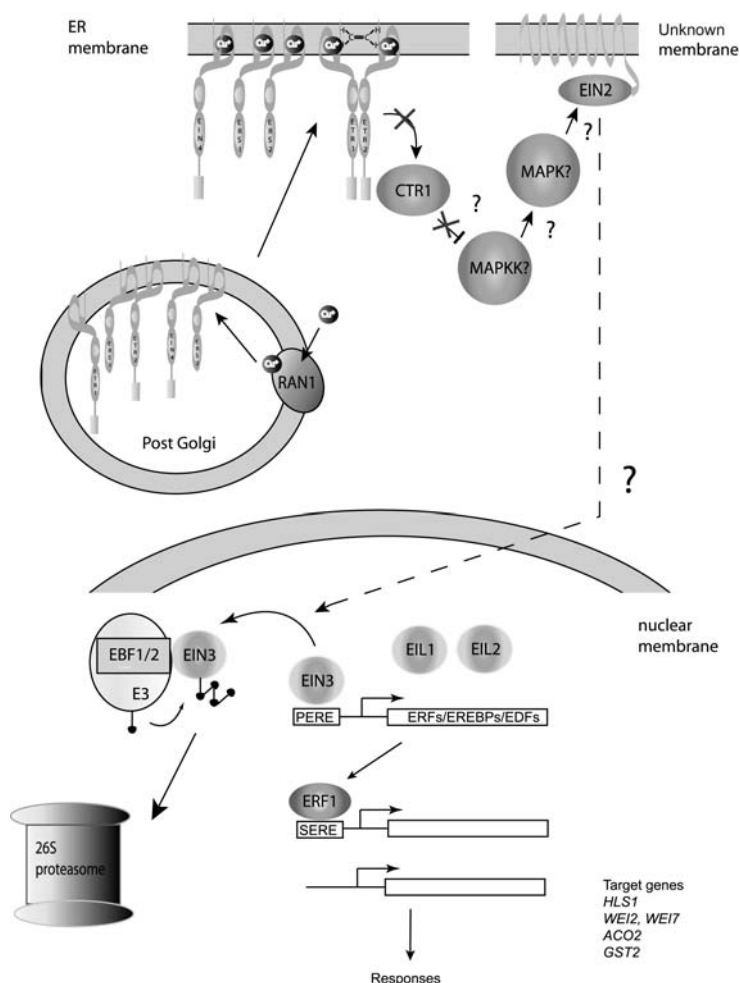


(Hua *et al.*, 1998). Furthermore, ethylene up-regulates expression of the ERS1, ERS2, and ETR2 receptor, which may be a desensitization response to prevent toxic effects of high levels of ethylene.

Yeast two-hybrid screens and *in vitro* binding assays have shown that both the kinase (transmitter) and the receiver domain of ETR1 and the kinase domain of ERS1 can directly interact with the downstream component CTR1 (Clark *et al.*, 1998) (Fig. 5.3). Physical interactions of the subfamily II ETR2 transmitter domain were also demonstrated, but are much weaker than those reported for ETR1 and ERS1 (Cancel & Larsen, 2002). These findings provide an explanation for the observation that subfamily I receptors play a particular role in ethylene signaling. They suggest that the difference between type I and type II receptors may lie in the strength of their physical interaction with CTR1, which would also explain why only *etr1 ers1* double loss-of-function mutants have a phenotype. Moreover, from studies of the missense *ctr1-8* mutant, it was concluded that the N-terminal region of CTR1 is necessary for interaction with the ethylene receptor ETR1, but has no detectable effect on the kinase activity of CTR1 (Huang *et al.*, 2003). Formation of an ETR1–CTR1 signaling complex was also observed *in planta*, where both proteins associate with the endoplasmic reticulum (Gao *et al.*, 2003). It was demonstrated that plants defective in multiple receptors cause detachment of CTR1 from the membrane. Moreover, the severity of the constitutive ethylene response in combined loss-of-function receptor mutants is correlated with the quantity of CTR1 present at the ER membrane. Considering the homology of CTR1 with MAPKKKs, its identification prompted the suggestion that an MAPK cascade may reside downstream of CTR1, and there are indications that such a cascade exists. Ouaked *et al.* (2003) showed that the MAPKK SIMKK specifically mediates ACC-induced activation of MAPKs in *Medicago*. Two MAPKs, SIMK and MMK3, were activated by ACC in this species, and two MAPKs are also activated in *Arabidopsis*, one of which is MPK6 and the second is a 44-kDa protein, which is probably MPK13. MPK6 may be a central player in ethylene-signaling downstream of CTR1 (Ouaked *et al.*, 2003). However, Liu and Zhang (2004) found that MPK6 affected ACS stability, rather than ethylene signaling.

Although CTR1 is highly important, ethylene-signal transduction is not entirely dependent on its activity. The *ctr1* null mutants are still capable of responding to ethylene (Larsen & Chang, 2001), and plants containing loss-of-function mutations in four ethylene receptors display a more severe phenotype than *ctr1* loss-of-function mutations (Hua & Meyerowitz, 1998). This leaves the way open for an additional branch in the pathway. The results of Hass *et al.* (2004) suggest that ARR2 may be part of an additional regulatory route. There may also be CTR1 homologues involved.

Further downstream EIN2 comes into play, as a positive regulator of ethylene signaling (Alonso *et al.*, 1999). Null *ein2* mutants cause strong ethylene insensitivity. The protein is similar to N-ramp metal transporter proteins with 12 membrane-spanning domains. Metal uptake in yeast could not be elicited by EIN2 expression, while this was the case for other N-ramp proteins (Thomine *et al.*, 2000). EIN2 is a



**Figure 5.3** Model for the ethylene-signal transduction chain in *Arabidopsis*. There are five ethylene receptors (ETR1, ERS1, ETR2, EIN4, and ERS2) which associate with the ER. Binding of ethylene is based on a co-ordination covalent bond to a copper atom integrated in the ethylene receptor apoproteins. Copper loading of ethylene receptors is performed by RAN1. The interaction of CTR1, an immediately downstream acting Raf-like kinase, with the receptors allows it to be localized to the ER. A MAPK module further passes on the signal. When CTR1 is inactivated by ethylene, MAPKK becomes activated and in turn activates the MAPK component in the cascade. The direct downstream targets of the MAPKs have yet to be determined. Inactivation of CTR1 results in the activation of EIN2, a membrane associated, positive regulator of ethylene responses, of which the actual signaling mechanism remains unknown. In the nucleus, an ethylene-dependent transcriptional cascade occurs. When activated by ethylene, members of the EIN3/EIL transcription factor family bind as dimers to the PERE in the promoters of primary response genes such as ETHYLENE-RESPONSE-FACTOR1 (ERF1). ERF1 and the other EREBPs bind to the GCC-box of secondary response targets, such as the defensin PDF1.2, hookless (HLS), and other ERFs, activating their transcription. EBF1 and -2 are F-box proteins that regulate ethylene signaling by targeting the EIN3 transcriptional regulator for ubiquitin-mediated degradation by the 26S proteasome.

component shared with several other signaling routes, including those for cytokinin, abscisic acid (ABA), and senescence. *EIN2* appears to be a single copy gene in a number of plant species, including rice, petunia, maize, and tomato (Gallie & Young, 2004; Jun *et al.*, 2004; Klee, 2004; Shibuya *et al.*, 2004).

From *EIN2*, the ethylene signal is transferred to the nucleus. At this point, there remains a hole in the maze. Although it has been shown that over-expression of the soluble C-terminus of *EIN2* provokes a constitutive triple response, it is not clear how the signal is passed from *EIN2* onto the downstream nuclear components (Alonso *et al.*, 1999), which include the *EIN3*, *EIN3*-like (*EIL*), and ERF transcription factors. The level of the *EIN3* protein is efficiently regulated by ethylene in an ubiquitin-dependent proteolysis process (Guo & Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004). In the absence of ethylene, *EIN3* is degraded through an ubiquitin/proteasome pathway mediated by the F-box proteins *EBF1* and *EBF2* (*EIN3*-binding F-box protein 1 and 2). Over-expression of *EBF1* resulted in ethylene insensitivity. In addition, *ebf1 ebf2* double loss-of-function mutants showed constitutive ethylene responses (Guo & Ecker, 2003; Potushak *et al.*, 2003), and in the case of strong alleles, growth arrest (Gagne *et al.*, 2004).

The stability of *EIN3* is a point for cross-talk with glucose signaling. Whereas ethylene inhibits the degradation of *EIN3*, glucose enhances it (Yanagisawa *et al.*, 2003). Of the five *EILs*, only *EIL1* has a functional overlap with *EIN3* (Chao *et al.*, 1997; Guo & Ecker, 2004).

*EIN3* is a transcription factor that forms the first step in a transcriptional cascade (Fig. 5.3). The *EIN3* proteins act as dimers (Solano *et al.*, 1998), and bind the promoter of Ethylene Response Factor 1 (*ERF1*) on a primary ethylene response element (PERE), with consensus sequence AYGWAYCT (Kosugi & Ohashi, 2000). Up until now and in spite of the identification of various *ERF1* homologues, the *ERF1* promoter is the only known *EIN3* target. *ERF1* itself is a member of a large family of Ethylene-Responsive Element-Binding Protein (EREBP) transcription factors (Alonso *et al.*, 2003b). However, only few of these EREBPs are ethylene regulated. It is therefore not surprising that they were found to function in a variety of processes. *ERF1* binds a secondary ethylene-responsive element (SERE) in promoters of target genes involved in pathogenesis, such as plant defensins (*PDF*). Moreover, *ERF1* is a common signaling component for another defence-related plant hormone, jasmonic acid (JA).

Large-scale transcriptome studies have added to the inventory of ethylene-regulated genes. In a genome-wide expression study, using an Affymetrix chip representing 22,000 *Arabidopsis* genes, Alonso *et al.* (2003b) identified four AP2 and B3 domain-containing proteins that were inducible by ethylene. They were designated *Ethylene response DNA-binding Factors* (EDF). Other microarray studies focused on different aspects of ethylene biology. Ethylene-regulated gene expression in *Arabidopsis* leaves was investigated using a cDNA microarray containing about 6000 unique genes (Van Zhong & Burns, 2003). In this study, the emphasis was on relatively long-term (24 h) ethylene regulation. For identification of genes involved in the very early phase of ethylene response, De Paepe *et al.* (2004) performed a kinetic analysis

of the transcriptional cascade by means of cDNA-AFLP and cDNA-microarray technology. They determined the major ethylene-regulated classes of genes. In particular, a large number of genes involved in cell rescue, disease, and defence mechanisms were identified as early ethylene-regulated genes, confirming the important role of ethylene in defence and stress responses.

## 5.4 A complex network

The current detailed view on ethylene biosynthesis and signaling pathways and their regulation paved the way to test interactions of specific players within these pathways with other endogenous or environmental cues. In recent years, evidence has accumulated that both ethylene biosynthesis and signaling are integrated in a broad network, in which some signals operate master switches while others take care of fine-tuning the output. Multiple hormones influence ethylene biosynthesis. Cytokinin treatment increases the stability of ACS5 (Chae *et al.*, 2003) so that many of the growth defects attributed to cytokinins are the result of ethylene overproduction (Vogel *et al.*, 1998a). In addition, auxin stimulates ethylene biosynthesis by raising ACS transcript levels (Abel *et al.*, 1995; Yamagami *et al.*, 2003). In mung bean, an extra increase in *VrACS6* and *VrACS7* mRNA was shown in response to brassinosteroid treatment, while *VrACS1* was down-regulated (Yi *et al.*, 1999). This suggests that ethylene biosynthesis is regulated by a delicate hormone-dependent mechanism.

Besides specific screens for ethylene mutants, alleles of mutations in ethylene signaling have also been recovered from screens for resistance to auxin transport inhibitors or to cytokinins, or for suppressors and enhancers of ABA-related mutants, or to uncover regulators of sugar metabolism (Vogel *et al.*, 1998b; Zhou *et al.*, 1998; Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). In addition to interactions with glucose and ABA at germination (Gazzarrini & McCourt, 2001), ethylene is often part of a network of hormonal interplay during vegetative plant development (Vandenbussche & Van Der Straeten, 2004; Vandenbussche *et al.*, 2005).

Auxin and ethylene co-ordinately regulate several developmental programmes in plants. For example, in *Arabidopsis* both hormones regulate root growth, root hair elongation, and hypocotyl phototropism (Pitts *et al.*, 1998; Harper *et al.*, 2000; Le *et al.*, 2001; Rahman *et al.*, 2001). Nevertheless, it is often unclear whether developmental effects attributed to auxin are solely due to this hormone or rather mediated by ethylene, or resulting from a synergistic interaction between both hormones.

Other developmental and growth response involves cross-talk between ethylene and gibberellins (GAs). The opposite response of terrestrial and semi-aquatic plants to ethylene is explained by the effect of ethylene on GA biosynthesis and signaling. Ethylene reduces cell elongation in dark-grown seedlings even in the presence of a high concentrations of GA. Molecular analysis demonstrated that cell

elongation is inhibited by the presence of a high level of RGA, a negative regulator of the GA-signaling pathway belonging to the family of DELLA proteins (Achard *et al.*, 2003; Vriezen *et al.*, 2004). Ethylene appears to modulate the GA signal by enhancing RGA stability. Root growth is also repressed by DELLA proteins, which are removed from the nucleus in the presence of the growth-promoting signals auxin or GA, presumably due to enhanced degradation (Fu & Harberd, 2003). This process is counteracted by ethylene, which stabilizes DELLA repressors in the nucleus, thus inhibiting root elongation.

Bipartite hormone interactions are most simple to consider, but may not reflect the actual situation *in planta*. More complex hormonal interactions have been demonstrated for the regulation of shoot meristem activity (Vandenbussche & Van Der Straeten, 2004). In addition, a classical auxin–ethylene interaction (Lehman *et al.*, 1996; Raz & Ecker, 1999), the formation and maintenance of the apical hook in *Arabidopsis*, was recently re-evaluated. A higher level of complexity was demonstrated, involving GAs and brassinosteroids, besides the previously defined players (Achard *et al.*, 2003; Alabadí *et al.*, 2004; Vriezen *et al.*, 2004; De Grauwe *et al.*, 2005). The current model suggests that ethylene stimulates brassinosteroid biosynthesis at the outer side of the apical hook. Brassinosteroids could regulate the transport of auxins to the inner side of the hook, thus creating supra-optimal levels of auxins. GAs are the most downstream components determining the severity of response.

Cross-talk with ethylene biosynthesis and signaling pathways also occurs in the pathogen defence response with JA and salicylic acid (SA) (Glazebrook, 1999). A microarray analysis suggested the co-ordination between these three signaling pathways, supported by a big overlap in gene expression, especially between jasmonate and ethylene (Schenk *et al.*, 2000). Previous studies have shown that both ethylene and JA are required for the induction of the defensin gene *PDF1.2* (Plant Defensin 1.2) in response to the avirulent fungal pathogen *Alternaria brassicicola* (Penninckx *et al.*, 1998). Furthermore, it was demonstrated that ethylene and jasmonate pathways converge at *ERF1*, the expression of which can be activated rapidly by ethylene or jasmonate, but also synergistically by both hormones (Lorenzo *et al.*, 2003). Moreover, blocking either pathway by mutations prevents *ERF1* induction by the two hormones either alone or in combination; therefore, both signaling pathways are required concurrently for the induction of *ERF1* expression and the activation of its target gene *PDF1.2*. These results suggest that *ERF1* acts downstream of the intersection between ethylene- and jasmonate-signaling pathways and that this transcription factor is a key element in the integration of both signals for the regulation of defence response genes.

In conclusion, almost all steps of ethylene biosynthesis and signaling are specifically controlled throughout development and serve as cross-roads with other signaling routes. An integration with the glucose, jasmonate, auxin, cytokinin, GA, and ABA pathways, but also with light and circadian clock signaling has been illustrated (Gazzarrini & McCourt, 2003; Leon & Sheen, 2003; Yanagisawa *et al.*, 2003; Vandenbussche & Van Der Straeten, 2004; Chae & Kieber, 2005; Rieu *et al.*, 2005; and chapters in this book). The puzzle of plant life is yet to be completed.

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## 6 Gibberellin metabolism and signal transduction

Stephen G. Thomas and Peter Hedden

### 6.1 Introduction

The term gibberellin was introduced by Teijiro Yabuta in 1935 for a growth active substance produced by the phytopathogenic fungus *Gibberella fujikuroi* (see Phinney, 1983, for a review of the early history of the gibberellins). Two crystalline substances were obtained from fungal extracts and named gibberellin A and B, the former being subsequently shown to be a mixture of three compounds, gibberellin A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub>. This system of nomenclature was later adopted for newly characterised gibberellins from all sources (MacMillan & Takahashi, 1968), and to date 136 different structures have been identified from higher plants, fungi or bacteria (MacMillan, 2002) (<http://www.plant-hormones.info/galinfo.htm>). It is now common practice to abbreviate gibberellin A<sub>n</sub> to GA<sub>n</sub> and to use GA as a general abbreviation for gibberellin. However, in this context, GA does not refer to gibberellic acid, which is an alternative name for GA<sub>3</sub>, the first gibberellin structure to be elucidated.

The ability of GA<sub>3</sub> to normalise the dwarf phenotype of certain mutants provided the first indication that GAs might be endogenous growth regulators in higher plants, and this was supported by the identification of GAs in seeds of runner bean almost 50 years ago. Such GA-deficient mutants have been largely instrumental in identifying the myriad developmental processes in which GAs participate, including seed germination, seedling growth, determination of leaf size and shape, stem and root extension, flower induction and development, pollination, seed development and fruit expansion. Although there was considerable excitement at the discovery of this new growth hormone, particularly in anticipation of its commercial exploitation, the lack of appropriate technology meant that progress in understanding GA signaling in plants was slow. An exception to this was  $\alpha$ -amylase induction in the cereal aleurone, which has long been the best characterised GA response, at least in terms of the downstream events (Jones & Jacobsen, 1991). Although some progress was made in understanding the chemical and biochemical details of the GA-biosynthetic pathways, knowledge of the signal transduction events was sparse. However, in the last 15 years, spurred on particularly by advances in molecular genetics, our knowledge of GA metabolism and signal transduction has advanced spectacularly, culminating in the recent identification of a soluble GA receptor (Ueguchi-Tanaka *et al.*, 2005). Furthermore, use of DNA microarrays is allowing the identification of many more downstream gene targets for the GA-signaling pathway and will lead to a clearer understanding of how the developmental consequences of GA action arise.



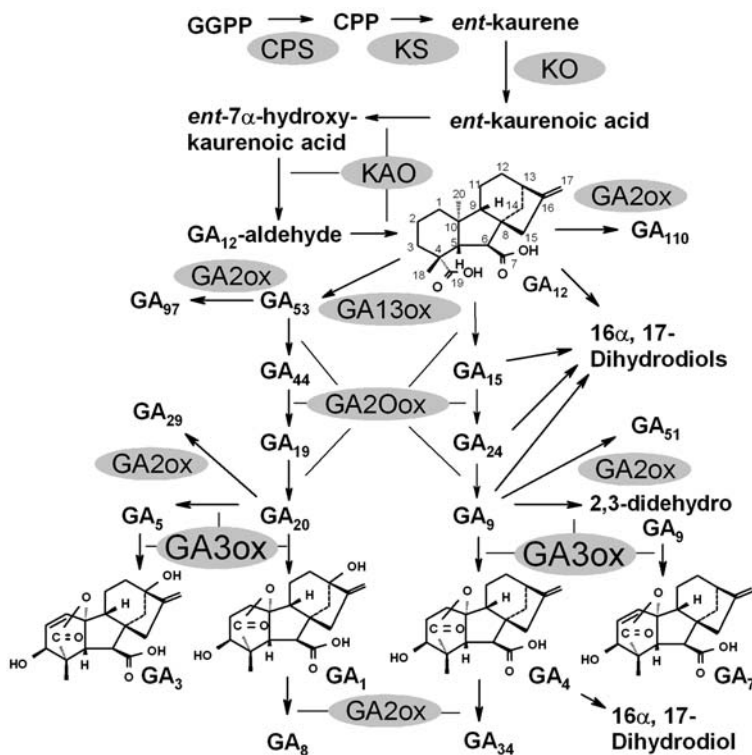
Our aim in this chapter is to highlight the more recent advances in GA metabolism and signal transduction, which can be considered as parts of a single signaling pathway. The closeness with which the two parts of the pathway are integrated is evident from feedback regulation of GA biosynthesis, which is mediated by GA-signaling transduction. We will also consider the regulation of GA-signaling by developmental and environmental stimuli, the transcriptional events that are targets of GA action as well as current information on the sites of GA biosynthesis and action.

## 6.2 The gibberellin metabolic pathway

### 6.2.1 Biosynthesis of bioactive GAs

The principal growth active structures GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub> and GA<sub>7</sub> are tetracyclic diterpenoid carboxylic acids possessing a 20-nor *ent*-gibberellane skeleton, a carboxyl group on C-6, a lactone function between C-4 and C-10, and a hydroxyl or other functionality at C-3 $\beta$ . Their biosynthesis, as outlined in Fig. 6.1, has been discussed in numerous recent reviews (Hedden & Phillips, 2000; Yamaguchi & Kamiya, 2000; Olszewski *et al.*, 2002; Sponsel & Hedden, 2004). Synthesis from the common diterpene precursor geranylgeranyl diphosphate (GGPP) involves the action of terpene cyclases, cytochrome P450 monooxygenases (P450s) and 2-oxoglutarate-dependent dioxygenases (2ODDs). In common with other diterpenes, GAs are produced predominantly via the plastid-localised methylerythritol phosphate (MEP) pathway in vegetative tissues, although there is a small contribution from the cytosolic mevalonic acid (MVA) pathway, presumably because of some movement of isopentenyl diphosphate (IPP) into plastids from the cytosol (Kasahara *et al.*, 2002). The relative contributions of the MEP and MVA pathways to GA biosynthesis may in fact vary between tissues, depending on the type of plastid present and the permeability of its envelope to IPP. Cyclisation of GGPP to the hydrocarbon intermediate *ent*-kaurene requires two enzymes, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase, which are thought to be located in the plastid (Sun & Kamiya, 1994; Helliwell *et al.*, 2001b). Surprisingly, it was shown recently that the relatively volatile *ent*-kaurene is readily released into and taken up from the atmosphere (Otsuka *et al.*, 2004), suggesting facile transport from the plastid, presumably via the endomembrane system. Although some species, such as the Japanese cedar, *Cryptomeria japonica*, produce large amounts of *ent*-kaurene that could in theory affect the growth of neighbouring plants, GA concentration is normally regulated late in the biosynthetic pathway so that applied *ent*-kaurene has relatively little effect on plant growth. Nevertheless, *ent*-kaurene-deficient mutants are extremely sensitive to this compound when it is present in the headspace of a sealed chamber (Otsuka *et al.*, 2004).

*ent*-Kaurene is converted to GA<sub>12</sub> by two cytochrome P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid hydroxylase (KAO). Transient expression of fusions of the enzymes with green fluorescent protein (GFP) indicate



and between tissues within the same species. Most species produce GA<sub>1</sub> as the major growth active GA in vegetative tissues, although *Arabidopsis* contains more GA<sub>4</sub>, and is more sensitive to this GA than to GA<sub>1</sub> (Desgagne-Penix *et al.*, 2005).

The GA 3-oxidases catalyse the final step in the formation of biologically active GAs. Recombinant GA3ox enzymes from *Arabidopsis* (AtGA3ox1) (Williams *et al.*, 1998) and pea (PsGA3ox1) (Lester *et al.*, 1997; Martin *et al.*, 1997) were shown to function as strict 3 $\beta$ -hydroxylases, converting GA<sub>9</sub> and GA<sub>20</sub> to GA<sub>4</sub> and GA<sub>1</sub>, respectively, as sole products. However, some GA 3-oxidases are less regiospecific and oxidise also at C-1 and C-2, resulting, for example, in the formation of GA<sub>5</sub>, by desaturation, and then GA<sub>3</sub>, by successive oxidation at C-1 $\beta$  and C-3 $\beta$  (Albone *et al.*, 1990). In an extreme example of low regiospecificity, a GA3ox from wheat was found to possess 13-hydroxylase activity, which requires that the substrate is turned through 180° at the enzyme active site (Appleford *et al.*, 2006).

### 6.2.2 GA deactivation

Effective regulation of the concentration of the bioactive hormone requires deactivation mechanisms, the best characterised of which is hydroxylation at the C-2 $\beta$  position. The enzymes responsible for this reaction (GA 2-oxidases) are 2ODDs that fall into three classes on the basis of their derived amino acid sequence: enzymes in the first two classes are specific for C<sub>19</sub>-GAs, oxidising the active hormones themselves or their non-3 $\beta$ -hydroxylated precursors (Thomas *et al.*, 1999; Elliott *et al.*, 2001), while enzymes of the third class accept only C<sub>20</sub>-GAs as substrates (Schomburg *et al.*, 2003; Lee & Zeevaart, 2005). One enzyme has been described from spinach that accepts C<sub>19</sub>- and C<sub>20</sub>-GA substrates, although it belongs to the first class of GA 2-oxidases (Lee & Zeevaart, 2002).

A second deactivation mechanism has been characterised recently in rice (Zhou *et al.*, 2005). The *ELONGATED UPPERMOST INTERNODE* gene, a mutant allele of which is used in rice breeding to ensure emergence of the panicle from the flag leaf, was shown to encode a cytochrome P450 that epoxidises the 16, 17-double bond. The enzyme converts non-13-hydroxylated C<sub>20</sub>- and C<sub>19</sub>-GAs to inactive products and may therefore act at several points in the pathway. Although 16, 17-epoxides could not be detected, even when the *EUI* gene was overexpressed, there was accumulation of 16 $\alpha$ , 17-dihydrodiols, presumably produced by hydration of the epoxides, either *in planta* or during extraction. Gibberellin 16, 17-dihydrodiols have been detected in many plants, where they often occur at relatively high concentrations, indicating that epoxidation may be a general deactivation mechanism. Elongation of the upper internode and an accumulation of GA<sub>4</sub> in the *eui* rice mutants demonstrate this mechanism may serve to control GA concentration in some tissues.

Deactivation may also result from GA conjugation, which involves mainly the formation of glucosyl ethers and esters (reviewed by Schneider & Schliemann, 1994). However, the most abundant glucosides consist of 2 $\beta$ -hydroxylated GAs coupled through their 2 $\beta$ -hydroxyl group so that their formation cannot have a role in deactivation, although it may serve to sequester GAs in the vacuole.

### 6.3 Genes of GA biosynthesis and their regulation

The availability of complete genome sequences for *Arabidopsis* and rice has enabled the identification of most of the genes involved in GA biosynthesis and deactivation in these species (Hedden *et al.*, 2001; Sakamoto *et al.*, 2004). However, the list of genes (Table 6.1) is not complete and genes encoding enzymes with novel functions in GA biosynthesis or previously unknown classes of the known enzymes are still likely to be discovered. A common feature is that enzymes catalysing early steps in the pathway are encoded by single or limited numbers of genes, while the 2ODDs are encoded by gene families, the members of which differ in their spatial and temporal patterns of expression. This is consistent with these later genes being the primary sites of regulation. In *Arabidopsis*, *CPS*, *KS* and *KO* are present as single copies, while there are two fully redundant *KAO* genes. Although there are four *CPS*-like genes in rice (Sakamoto *et al.*, 2004), only one, *OsCPS1*, appears to be involved in GA biosynthesis (Otomo *et al.*, 2004). Similarly, mutant studies indicate that only one member from each of the nine-member *KS*-like and four-member *KO*-like gene families has a major role in GA production in rice (Sakamoto *et al.*, 2004). In contrast to *Arabidopsis*, rice has a single *KAO* gene. Null mutations in these early genes cause severe pleiotropic phenotypic abnormalities, such as extreme stunting, that are characteristic of GA deficiency whereas the effects from loss of a functional 2ODD gene are much less severe, indicating that the paralogues are partially redundant, as a result of overlapping expression patterns or movement of intermediates between tissues.

GA biosynthesis and deactivation are regulated by numerous developmental and environmental factors, much of this regulation acting on the 2ODDs, the activity of which have a major influence on GA content. This is illustrated for the GA 20-oxidase in *Arabidopsis* by work with transgenic plants. Overexpression of a *GA20ox* gene caused increased GA<sub>4</sub> content, accelerated bolting and longer stems (Huang *et al.*, 1998; Coles *et al.*, 1999), whereas increasing expression of *CPS* and *KS* resulted in higher amounts of *ent*-kaurene and GA<sub>12</sub>, but had no effect on the levels of bioactive GAs or the phenotype (Fleet *et al.*, 2003).

#### 6.3.1 Developmental regulation

Expression of *CPS*, which encodes the enzyme that catalyses the first committed step in GA biosynthesis and might be expected to control the flux into the pathway, shows strong developmental regulation. On the basis of experiments with a promoter–GUS reporter gene in *Arabidopsis*, *CPS* was shown to be expressed most strongly in growing organs or in vascular tissues, consistent with the presence of the enzyme in immature plastids rather than chloroplasts (Silverstone *et al.*, 1997a). Positive regulation within these tissues required the presence of the second intron and the promoter region from –1391 to –997 upstream of the translation initiation site (Chang & Sun, 2002). The expression of *CPS* in vascular tissues is consistent with the findings of Ross *et al.* in 2003 (and Chapter 9) from work with pea

**Table 6.1** A comparison of GA-metabolic genes in *Arabidopsis* and rice

Enzyme	<i>Arabidopsis</i>				Rice			
	Gene name	<i>Arabidopsis</i> locus	AGI locus identifier	References	Gene name	Rice locus	Accession number	References
<i>ent</i> -Copalyl diphosphate synthase	<i>AtCPS</i>	<i>GA1</i>	At4g02780	(1)	<i>OsCPS</i>		AP004872	(2)
<i>ent</i> -Kaurene synthase	<i>AtKS</i>	<i>GA2</i>	At1g79460	(3)	<i>OsKS</i>		OSJN00255	(2)
<i>ent</i> -Kaurene 19-oxidase	<i>AtKO</i>	<i>GA3</i>	At5g25900	(4)	<i>OsKO</i>	<i>D35</i>	AP005471	(5)
<i>ent</i> -Kaurenoic acid oxidase	<i>AtKAO1</i>		At1g05160	(6)	<i>OsKAO</i>		AP000616	(2)
	<i>AtKAO2</i>		At2g32440	(6)				
GA 20-oxidase	<i>AtGA20ox1</i>	<i>GA5</i>	At4g25420	(7)	<i>OsGA20ox1</i>		AC096690	(8)
	<i>AtGA20ox2</i>		At5g51810	(7)	<i>OsGA20ox2</i>	<i>SD1</i>	AP003561	(9)
	<i>AtGA20ox3</i>		At5g07200	(7)	<i>OsGA20ox3</i>		AP005840	(2)
	<i>AtGA20ox4</i>		At1g60980	(10)	<i>OsGA20ox4</i>		AC124836	(2)
	<i>AtGA20ox5</i>		At1g44090	(10)				
GA 3-oxidase	<i>AtGA3ox1</i>	<i>GA4</i>	At1g15550	(12)	<i>OxGA3ox1</i>		AC144738	(13)
	<i>AtGA3ox2</i>		At1g80340	(14)	<i>OsGA3ox2</i>	<i>D18</i>	AP002523	(13)
	<i>AtGA3ox3</i>		At4g21690	(10)				
	<i>AtGA3ox4</i>		At1g80330	(10)				
GA 2-oxidase* (I)	<i>AtGA2ox1</i>		At1g78440	(15)	<i>OsGA2ox3</i>		AP003375	(16)
	<i>AtGA2ox2</i>		At1g30040	(15)	<i>OsGA2ox4</i>		AC132485	(2)
	<i>AtGA2ox3</i>		At2g34555	(15)				
(II)	<i>AtGA2ox4</i>		At1g47990	(10)	<i>OsGA2ox1</i>		AC119288	(17)
	<i>AtGA2ox6</i>		At1g02400	(10,16)	<i>OsGA2ox2</i>		AP003143	(16)
(III)	<i>AtGA2ox7</i>		At1g50960	(19)	<i>OsGA2ox5</i>		AP005187	(20)
	<i>AtGA2ox8</i>		At4g21200	(19)	<i>OsGA2ox6</i>		OSJN00156	(20)

References: (1) Sun and Kamiya (1994); (2) Sakamoto *et al.* (2004); (3) Yamaguchi *et al.* (1998); (4) Helliwell *et al.* (1998); (5) Itoh *et al.* (2004); (6) Helliwell *et al.* (2001); (7) Phillips *et al.* (1995); (8) Toyomasu *et al.* (1997); (9) Spielmeier *et al.* (2002); (10) Hedden and Phillips (2000); (11) Hedden *et al.* (2001); (12) Chiang *et al.* (1995); (13) Itoh *et al.* (2001); (14) Yamaguchi *et al.* (1998); (15) Thomas *et al.* (1999); (16) Sakai *et al.* (2003); (17) Sakamoto *et al.* (2001); (18) Wang *et al.* (2004); (19) Schomberg *et al.* (2003); (20) Lee and Zeevaert (2005).

\* The GA 2-oxidase family divides into three different clades (I, II and III). Members of class III can 2 $\beta$ -hydroxylate only C<sub>20</sub>-GAs (the activities of OsGA2ox5/6 have not been characterised).

seedlings that mature tissues are capable of *de novo* GA biosynthesis. They suggest that high rates of GA 2-oxidation in such tissues accounts for their low concentrations of biologically active GAs compared with developing tissues.

The identification of transcription factors that interact with the promoters of GA-biosynthetic genes is providing clues to the molecular mechanisms by which the spatial and temporal expression of these genes is regulated. The most extensively-studied factors are the KNOTTED1-like homoeobox (KNOX) proteins, which are homoeodomain transcription factors that are implicated in the establishment of meristem identity as well as in determining leaf morphology. KNOX proteins repress GA biosynthesis by suppressing GA 20-oxidase gene expression (Sakamoto *et al.*, 2001a; Hay *et al.*, 2002; Rosin *et al.*, 2003). The tobacco KNOX protein NTH15 was shown to bind to *cis* elements within the promoter and first intron of a tobacco *GA20ox* gene, with stronger binding to the intron sequence (Sakamoto *et al.*, 2001a). KNOX proteins interact with a second class of homoeodomain proteins, the BEL1-like proteins, and the two proteins appear to function as heterodimers (Smith *et al.*, 2002). Both types of transcription factors suppressed expression of a *GA20ox* gene in potato, while the heterodimer was most effective and bound most strongly to the promoter (Chen *et al.*, 2004). In this case, only the BEL protein bound to the intron.

*KNOX* genes are expressed in the meristem in vegetative apices of tobacco and *Arabidopsis*, such that *GA20ox* expression is excluded from this region but occurs in the leaf primordia at the flanks of the meristem and in the sub-apical region (Sakamoto *et al.*, 2001a; Hay *et al.*, 2002). In tomato and potato *KNOX* genes are also expressed in the leaf primordia, where they function in the establishment of leaf morphology (Hay *et al.*, 2002; Rosin *et al.*, 2003). KNOX has also been shown to enhance expression of the *Arabidopsis* genes *GA2ox2* and *GA2ox4*, which, on the basis of GUS expression in reporter lines, is located at the base of the meristem and may offer protection from the influx of GAs from surrounding leaves (Jasinski *et al.*, 2005). Since KNOX proteins also stimulate cytokinin biosynthesis by promoting expression of *IPT* genes (Jasinski *et al.*, 2005; Yanai *et al.*, 2005), these proteins function by establishing a favourable hormone balance, high cytokinin and low GA, for maintaining meristem identity. The homoeotic gene *AGAMOUS* (AG), which is activated in the meristem after flower induction, induces expression of *AtGA3ox1* (Gomez-Mena *et al.*, 2005). This function may serve to increase GA production within the meristem and thereby promote cell differentiation and initiate production of floral organs.

The LEC3 and FUS3 transcription factors function to promote embryogenesis in *Arabidopsis*. Both factors were shown to suppress expression of *AtGA3ox2* and thereby reduce GA biosynthesis during early embryo development (Curaba *et al.*, 2004). FUS3 bound directly to two RY elements in the promoter of *AtGA3ox2*. Consistent with early embryogenesis requiring a low GA regime, the MADS domain protein AGAMOUS-like 15 (AGL15), which accumulates during embryo development, enhanced expression of a gene, *AtGA2ox6*, involved in GA deactivation (Wang *et al.*, 2004). In this case too it was possible to identify target sites for AGL15 on the *AtGA2ox6* promoter.

A bZIP transcription factor from tobacco, known as RSG (repression of shoot growth) was shown to bind and activate the promoter of the *Arabidopsis*



*ent*-kaurene oxidase (*KO*) gene (Fukazawa *et al.*, 2000). A dominant negative form of RSG, which retains DNA binding but no longer activates expression, inhibited GA biosynthesis and stem extension in tobacco consistent with the function of RSG in promoting GA production. In addition, the dominant negative RSG appeared to interfere with feedback regulation of a *GA20ox* gene (see later) so may also bind to the promoter of this gene (Ishida *et al.*, 2004). Gibberellin activity leads to exclusion of RSG from the nucleus via phosphorylation-mediated binding to a 14-3-3 protein that sequesters it in the cytoplasm (Ishida *et al.*, 2004). This would provide a mechanism for feedback regulation of *GA20ox* expression, although dominant negative RSG had no effect on the feedback regulation of a *GA3ox* gene and, furthermore, there is no evidence that *KO* is feedback regulated. The physiological function of RSG is still not clear.

### 6.3.2 Hormonal regulation

Gibberellin homeostasis is maintained by regulation of its own metabolism via the DELLA protein-dependent signaling pathway, as discussed in Section 6.5.2. There is evidence that other plant hormones also modify GA metabolism, allowing integration between different hormone signaling pathways. The best characterised interaction is with auxin, the action of which results in increased GA concentrations. In some systems, such as pod set (van Huizen *et al.*, 1997) and internode elongation (O'Neill & Ross, 2002) in pea, tissue elongation in response to auxin is dependent on GA, which acts as a secondary messenger for the long-distance signal provided by auxin. Regulation of GA biosynthesis in the pod is specific for 4-chloro-IAA, which originates from the seed and enhances expression of *PsGA20ox1* and *PsGA3ox1* (Ngo *et al.*, 2002; Ozga *et al.*, 2003) in the pod, while in the stem, IAA from the shoot apex induces *PsGA3ox1* expression and suppresses expression of *PsGA20ox1* (O'Neill & Ross, 2002). Evidence for auxin regulation of GA biosynthesis in other species suggests that this is a general mechanism, although the targets may vary between species. On the basis of GA measurements and metabolism experiments it was shown that auxin from the developing barley inflorescence regulated stem elongation by promoting GA 3-oxidation and suppressing 2-oxidation in internodes and nodes (Wolbang *et al.*, 2004), while in tobacco internodes GA 20-oxidase is the major target for auxin from the shoot apex (Wolbang & Ross, 2001). In tobacco, auxin also suppresses GA deactivation by 2-oxidation. Evidence for regulation of GA metabolism by other hormones is less convincing. Although Bouquin *et al.* (2001) found that *AtGA20ox1* expression was increased by application of epibrassinolide to an *Arabidopsis* brassinosteroid-deficient mutant, Jager *et al.* (2005) could find no evidence that brassinosteroids function through altering GA metabolism in pea.

### 6.3.3 Environmental regulation

An important function of GAs is to act as intermediaries between environmental signals and resulting changes in growth and developmental patterns. The induction of developmental processes, such as seed germination, de-etiolation or flower formation



in response to light, photoperiod or cold have been shown to involve, at least in part, changes in GA concentration due to effects of the environmental cues on GA biosynthesis and deactivation. Stimulation of seed germination in lettuce (Toyomasu *et al.*, 1998) and *Arabidopsis* (Yamaguchi *et al.*, 1998a) by red light is associated with increased synthesis of bioactive GAs in the embryo. As well as stimulating the elongation of the radicle and hypocotyl, GA induces the synthesis of enzymes that digest the endosperm and allow the radicle to emerge. In both species, red light induces expression of *GA3ox* genes, *LsGA3ox1* in lettuce and *AtGA3ox1* and *AtGA3ox2* in *Arabidopsis*. The induction is reversed by irradiation with far-red (FR) light, indicating the involvement of phytochromes in this process. Although the kinetics of induction of the two *Arabidopsis* genes is similar, their subsequent developmental patterns of expression differ markedly: the increase in *AtGA3ox1* expression is transient, although expression is restored after about 36 h and remains high throughout seedling development, while *AtGA3ox2* expression is associated only with germination, remaining high for about 36 h and then dropping to low levels (Yamaguchi *et al.*, 1998a). Expression of *AtGA3ox1*, but not that of *AtGA3ox2*, is also enhanced by exposure of imbibed *Arabidopsis* seeds to low temperatures (Yamauchi *et al.*, 2004). Stimulation of germination by this treatment, a process known as stratification, is dependent on the presence of *AtGA3ox1*, clearly demonstrating the involvement of GAs in this process. Recently it was shown that regulation of *GA3ox* gene expression by light and temperature in *Arabidopsis* seed is mediated by the basic helix–loop–helix transcription factor SPATULA (SPT) in concert with the related protein PIL5 (Penfield *et al.*, 2005).

In contrast to the stimulatory effect of red light on *GA3ox* expression in photoblastic seeds, exposure of dark-grown pea seedlings to red light results in a rapid reduction in *PsGA3ox1* expression, accompanied by an increase in *PsGA2ox2* expression (Reid *et al.*, 2002). About 4 h after exposure to light, *PsGA3ox1* and *PsGA2ox2* transcripts in the de-etiolating pea shoots return to initial values and there is an increase in *PsGA20ox1* expression, presumably as a response to the reduced GA concentration. Since GA action suppresses many facets of photomorphogenesis (Alabadi *et al.*, 2004) reducing GA content is necessary for full de-etiolation.

The photoreceptor for suppression of *PsGA3ox1* expression in pea is PHYA and possibly also a blue light receptor (Reid *et al.*, 2002), whereas photostable phytochromes are involved in *GA3ox* induction in seeds (Yamaguchi *et al.*, 1998a). A blue light receptor has also been invoked for induction of *GA20ox* expression by light in potato (Jackson *et al.*, 2000). Higher levels of expression of the *StGA20ox1* gene occur in the light than in the dark. Lines with reduced PHYB content contained higher levels of *StGA20ox1* transcript suggesting that this receptor may also regulate expression of the gene, although this regulation did not apparently account for the light–dark difference (Jackson *et al.*, 2000). Although tuber formation in potatoes is controlled by photoperiod and is inhibited by GA, the involvement of the light regulation of GA biosynthesis in this process is not clear since there was no difference in *GA20ox* expression or of GA content between plants grown in long (LD) and short days (SD).

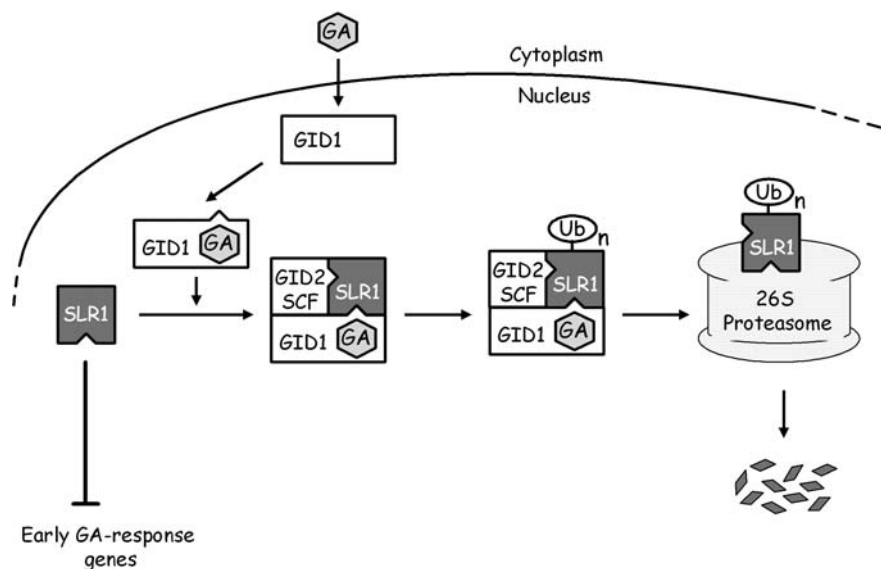
However, there are several reports of higher expression of *GA20ox* genes in LD compared to SD in rosette species (Wu *et al.*, 1996; Xu *et al.*, 1997; Lee & Zeevaart, 2002). Flowering in such species is sensitive to photoperiod and is accompanied by stem extension (bolting), a process that requires GA activity. In spinach, which has an absolute LD requirement for flowering, transfer of plants from SD to LD resulted in increased concentrations of C<sub>19</sub>-GAs associated with higher levels of expression of *SoGA20ox1*, particularly in petioles and leaf tips (Wu *et al.*, 1996; Lee & Zeevaart, 2002). Expression of a GA 2-oxidase gene, *SoGA20ox1* was reduced by LD in young leaves and tips, but increased in petioles, perhaps due to feedforward regulation, so it may not be under photoperiod control in this tissue (Lee & Zeevaart, 2002). Indeed, the increased expression of *SoGA20ox1* in petioles may be due to light induction rather than circadian regulation since prolonged exposure to light caused high levels of expression regardless of the previous photoperiod. Petiole elongation in *Arabidopsis* is regulated by two *GA20ox* genes, expression of one of these, *AtGA20ox2*, is strongly induced by FR-rich light via the mediation of PHYB, but does not show diurnal oscillation (Hisamatsu *et al.*, 2005). Expression of the second gene *AtGA20ox1* was less strongly increased by exposure to FR, and showed diurnal oscillation so may thus be under circadian regulation. Blazquez *et al.* (2002) showed also that *AtGA20ox1* expression was controlled by the circadian clock from work with the circadian mutant *toc1*. However, while *AtGA20ox1* expression is indeed higher in LD (Xu *et al.*, 1997), the resulting increase in GA production may be related more to the stem extension that accompanies flowering than to the induction of flowering itself (Blazquez *et al.*, 2002). Balzquez *et al.* concluded that circadian regulation of GA biosynthesis and flowering time were independent. Gibberellins are, however, essential for flowering under non-inductive conditions (Wilson *et al.*, 1992). The grass *Lolium perenne* requires both vernalisation and LD for flowering, with the LD requirement, but not that for vernalisation, replaceable by application of GA. Exposure to LD caused upregulation of the 20-oxidase gene *LpGA20ox1* and increased GA signaling consistent with the photoperiod signal acting through GA in this case (MacMillan *et al.*, 2005). The involvement of GAs in flower induction in the related species *Lolium temulentum* is discussed in Section 6.6.3 and Chapter 10.

Plants have the ability to discriminate between day and night temperatures, a process known as thermoperiodism. Stem growth is affected by diurnal temperature differences, promoted by positive (higher in the day) and inhibited by negative (higher in the night) differential temperatures, in a GA-regulated process (Grindal *et al.*, 1998). It has been demonstrated in pea that the low growth rate in negative differential temperatures was associated with increased expression of the *PsGA20ox2* gene, suggesting a higher rate of GA deactivation (Stavang *et al.*, 2005). Plants also reduce their growth in response to stress and there is a negative correlation between GA content and stress tolerance (e.g. see Sarkar *et al.*, 2004). Evidence is now emerging that stress results in modified GA biosynthesis. Overexpression of *DDF1*, which encodes an AP2-like transcription factor that is closely related to the DREB (Dehydration Responsive Element Binding protein) factors involved in stress

responses, caused reduced GA content and dwarfism in *Arabidopsis* (Magome *et al.*, 2004). Expression of *DDF1* was strongly induced by salt stress while plants overexpressing this gene had increased stress tolerance. Based on the effects of *DDF1*-expression on GA content it was suggested that GA 20-oxidase was the target for DDF1, but this needs to be confirmed.

## 6.4 The gibberellin signal transduction pathway

In recent years there have been impressive advances in our understanding of how the GA-signal is transduced, subsequently leading to changes in GA-responsive growth and development. Studies in this field have in particular emphasised the central role played by the DELLA proteins, which function as repressors of GA-mediated responses (Thomas & Sun, 2004). However, until recently, the components responsible for perceiving bioactive GAs had remained elusive. An exciting recent study by Ueguchi-Tanaka *et al.* (2005) has now resulted in the identification of a soluble GA receptor from rice. The current model of GA-signal transduction, based on components identified in rice, is outlined in Fig. 6.2. The evidence to support this model is discussed below. Genes encoding signaling components, identified in *Arabidopsis* and rice, are listed in Table 6.2.



**Figure 6.2** *GA-signal transduction in rice.* In the absence of bioactive GAs, the DELLA protein, SLR1 acts as a repressor of GA responses. Gibberellins produced in, or transported to the cell bind to the nuclear localised GID1, promoting its association with SLR1. Through an unknown mechanism, this GA-dependent interaction causes the recognition of SLR1 by a SCF<sup>GID2</sup> Ub E3 ligase, resulting in its ubiquitination. The ubiquitinated SLR1 is then degraded by the 26S proteasome. Gibberellin responses are induced as a consequence of reduced levels of the SLR1 repressor.

**Table 6.2** A comparison of GA-signaling genes in *Arabidopsis* and rice

Signaling component	Protein type and function	GA-response regulator	<i>Arabidopsis</i>			Rice		
			Gene name	AGI locus identifier	References	Gene name	Accession number	References
GID1* DELLA	HSL-related protein. Soluble GA receptor	Positive				<i>GID1</i>	AB211399	(1)
	GRAS protein. Putative transcriptional regulator	Negative	<i>RGA</i>	At2g01570	(2)	<i>SLR1</i>	AB030956	(3)
			<i>GAI</i>	At1g14920	(4)			
			<i>RGL1</i>	At1g66350	(5,6)			
			<i>RGL2</i>	At3g03450	(5,7,8)			
SLY1/GID2	F-box protein. Component of an SCF Ub E3 ligase involved in targeting DELLA proteins for degradation	Positive	<i>RGL3</i>	At5g17490	(5)			
			<i>SLY1</i>	At2g24210	(9)	<i>GID2</i>	AB100246	(10)
			<i>SNE</i>	At5g48170	(11)			
SPY	O-GlcNAc transferase	Negative	<i>SPY</i>	At3g11540	(12)			
D1/GPA1	Heterotrimeric G-protein $\alpha$ subunit	Positive	<i>GPA1</i>	At2g26300	(13,14)	<i>D1</i>	D38232	(15,16)

References: (1) Ueguchi-Tanaka *et al.* (2005); (2) Silverstone *et al.* (1998); (3) Ogawa *et al.* (2000); (4) Peng *et al.* (1997); (5) Dill and Sun (2001); (6) Wen and Chang (2002); (7) Peng *et al.* (2002); (8) Tyler *et al.* (2004); (9) McGinnis *et al.* (2003); (10) Sasaki *et al.* (2003); (11) Strader *et al.* (2004); (12) Jacobsen *et al.* (1996); (13) Ma *et al.* (1990); (14) Ullah *et al.* (2001); (15) Ishikawa *et al.* (1995); (16) Ashikari *et al.* (1999).

Yamaguchi *et al.* (1998) (GA2); Helliwell *et al.* (1998); Helliwell *et al.* (2001); Toyomasu *et al.* (1997); Speilmeyer *et al.* (2002); Itoh *et al.* (2001); Sakai *et al.* (2003); Ogawa *et al.* (2000); Mat *et al.* (1990); Ishikari *et al.* (1995).

\*In *Arabidopsis* there are three genes that are homologous to *GID1*: *At3g63010*, *At3g05120* and *At5g27320*.

### 6.4.1 *The gibberellin receptor*

Biochemical studies have suggested the presence of both soluble cytoplasmic and plasma membrane-localised GA receptors in plants, but the necessary components could not be identified (Nakajima *et al.*, 1997; Lovegrove *et al.*, 1998). The identification and characterisation of rice *GA-insensitive dwarf* (*gid*) mutants has proved instrumental in elucidating the GA-signaling cascade (Sasaki *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005). The *gid1* mutants display a severe GA-insensitive dwarf phenotype, including defects in feedback regulation of GA biosynthesis that result in the accumulation of much higher levels of bioactive GAs than wild-type plants (Ueguchi-Tanaka *et al.*, 2005). The *gid1* mutations are recessive, indicating that *GID1* encodes a positive regulator of GA signaling. This is further supported by the demonstration that transgenic plants overexpressing *GID1* exhibit a GA-hypersensitive phenotype.

The *GID1* gene was isolated by positional cloning and demonstrated to encode a polypeptide of 354 amino acids with significant homology with members of the Hormone Sensitive Lipase (HSL) family (Ueguchi-Tanaka *et al.*, 2005). It appears unlikely that *GID1* possesses HSL activity because a histidine residue, essential for HSL activity (Osterlund, 2001) is replaced by a valine, and indeed recombinant *GID1* lacked HSL activity *in vitro*. Biochemical and genetic evidence indicates strongly that *GID1* functions as a GA receptor. Thus, recombinant *GID1* bound a radiolabelled, bioactive GA analogue, 16,17-dihydro[<sup>3</sup>H<sub>4</sub>]GA<sub>4</sub>, with both high affinity and rapid-binding kinetics. This binding was competed more effectively by bioactive GAs than by inactive compounds. Furthermore, the three *gid1* loss-of-function alleles, *gid1-1*, *-2* and *-3* produce mutant proteins that are no longer capable of binding GAs.

Localisation studies suggest that *GID1* exists as a predominantly soluble nuclear protein and is thus different from the proposed plasma membrane receptors involved in the induction of  $\alpha$ -amylase expression in cereal aleurone cells (Hooley *et al.*, 1992; Gilroy & Jones, 1994). It is possible that there are additional membrane localised GA receptors, although the observation that *gid1* mutants are insensitive to GA induction of  $\alpha$ -amylase expression does not support their existence in rice. Further insights will be provided by the characterisation of *GID1* orthologues in other cereals.

### 6.4.2 *DELLA proteins act as repressors of GA signaling*

The use of molecular genetic approaches in monocots and dicots has demonstrated the central role of the DELLA transcriptional regulators in GA-signal transduction. The DELLA proteins, so-called after a conserved N-terminal domain, act as repressors of the GA-signal transduction cascade (Olszewski *et al.*, 2002), as illustrated by the promotion of GA-independent growth in DELLA loss-of-function mutations (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Dill & Sun, 2001; Ikeda *et al.*, 2001; Gubler *et al.*, 2002). In contrast, DELLA gain-of-function mutations, which are semi-dominant, produce GA-insensitive dwarf plants due to the formation of constitutively active repressors that are unresponsive to the GA-signal (Peng *et al.*, 1997; Peng *et al.*, 1999; Dill *et al.*, 2001; Gubler *et al.*, 2002).

The DELLA proteins form a sub-group of the GRAS (named after the GAI, RGA and SCR members) family of transcriptional regulators (Pysh *et al.*, 1999). Members of the GRAS protein family have a highly conserved C-terminal domain, which appears to have a functional role. In the case of DELLA proteins, this is demonstrated by the observations that missense mutations in the GRAS (C-terminal) domain often result in loss-of-function (Silverstone *et al.*, 1998; Ikeda *et al.*, 2001; Gubler *et al.*, 2002). The mode of action of GRAS proteins is still unclear, although the demonstration that they are predominantly nuclear localised supports their role as transcriptional regulators (DiLaurenzio *et al.*, 1996; Silverstone *et al.*, 1998; Silverstone *et al.*, 2001; Fleck & Harberd, 2002), despite not containing a recognisable DNA-binding domain. The N-terminal domain of DELLA proteins is unique to this sub-group of the GRAS family and defines their role in GA signaling. It contains two highly conserved motifs, which have been designated domains 1 and 2 (Peng *et al.*, 1999). In most cases, DELLA gain-of-function mutations result in truncations, deletions or substitutions within these domains, which are, therefore, assumed to have an important regulatory role in GA signaling (Peng *et al.*, 1997; Peng *et al.*, 1999; Dill *et al.*, 2001; Gubler *et al.*, 2002; Itoh *et al.*, 2002).

Recent studies have highlighted the conserved role of DELLA proteins in GA signaling. However, a striking difference between monocots and dicots that has become apparent from studies in rice and *Arabidopsis* is the level of redundancy in dicots. Genome sequencing projects have confirmed that there are five DELLA genes (*GAI*, *RGA*, *RGL1*, *RGL2* and *RGL3*) in *Arabidopsis* whereas rice contains only one, *SLR1* (Ikeda *et al.*, 2001). Characterisation of the *slr1* null alleles indicates that SLR1 represses all GA responses. Although *SLR1* is considered to be the only DELLA gene responsible for mediating GA signaling in rice, searches of the rice genome databases have identified two highly homologous sequences, designated *SLR1-like-1* and *-2* (*SLRL1* and *-2*; Itoh *et al.*, 2005b). The C-terminal GRAS domains of SLRL1 and *-2* display strong sequence similarities with that of SLR1; however, neither contain the conserved N-terminal DELLA domain. Itoh *et al.* (2005a & b) provide some evidence that SLRL1 may function as a negative regulator of GA signaling in rice. However, it is clear that further studies, including reverse genetics approaches, are necessary to confirm such a role.

The five DELLA (*RGL*) genes in *Arabidopsis* display both overlapping and independent roles in repressing tissue-specific aspects of GA response. The physiological roles of individual *RGL* genes has been studied by observing the effects of *RGL* null alleles in suppressing the growth defects in the GA-deficient mutant, *gal-3* (Silverstone *et al.*, 1997b; Silverstone *et al.*, 1998; Dill & Sun, 2001; King, K.E. *et al.*, 2001; Lee *et al.*, 2002; Cheng *et al.*, 2004; Tyler *et al.*, 2004). *GAI* and *RGA* are the predominant DELLAs regulating vegetative development and floral initiation (Silverstone *et al.*, 1997b; Dill & Sun, 2001; King, K.E. *et al.*, 2001), while *RGA*, *RGL1* and *RGL2* are responsible for modulating floral development (Cheng *et al.*, 2004; Tyler *et al.*, 2004). Results on the identity of the DELLA proteins involved in germination are conflicting, although *RLG2* appears to have a major role (Lee *et al.*, 2002; Wen & Chang, 2002; Tyler *et al.*, 2004). Nevertheless, high

levels of *RGA*, *GAI* and *RGL3* transcripts are present in germinating seeds (Tyler *et al.*, 2004) so it is conceivable that *RGA*, *GAI* and *RGL3* participate in different developmentally or environmentally regulated aspects of seed germination. Supporting evidence for this hypothesis is provided by a recent study demonstrating that *RGL2*, *RGA* and *GAI* are required to repress germination in the dark (Cao *et al.*, 2005). More information on the cellular localisation of the *RGL* proteins should provide further clues to the developmental processes that they control.

#### 6.4.3 *GAs promote rapid degradation of DELLA proteins*

A significant breakthrough in understanding the function of DELLA proteins in GA signaling was the observations that GA promotes their degradation. The levels of a GFP:*RGA* fusion protein were rapidly reduced in the roots of transgenic *Arabidopsis* plants after treatment with bioactive GA, while application of the GA-biosynthesis inhibitor, paclobutrazol, increased GFP:*RGA* levels (Silverstone *et al.*, 2001). The endogenous *RGA* protein showed a similar response to GA treatment, which was not caused by a reduction in *RGA* transcripts. In fact, *RGA* transcript levels are slightly elevated by GA treatment (Silverstone *et al.*, 1998; McGinnis *et al.*, 2003). The GA-induced destabilisation of DELLA proteins is conserved across plant species and has been demonstrated, for example, in rice and barley (Gubler *et al.*, 2002; Itoh *et al.*, 2002). Furthermore, in *Arabidopsis*, a similar response is observed for the *RGA* homologues *GAI* and *RGL2* (Dill *et al.*, 2004; Fu *et al.*, 2004; Tyler *et al.*, 2004; Hussain *et al.*, 2005). The kinetics of GA-induced DELLA protein degradation is extremely rapid, with reduction in the levels of *SLN1* and *RGA* being observed in barley and *Arabidopsis*, respectively, within 10 min of GA treatment (Gubler *et al.*, 2002; Sun and Thomas, unpublished observations). Thus the role of GA is to target DELLA proteins for degradation and thereby relieve their repression of growth.

Gain-of-function mutations in DELLA genes have provided some important insights into the functional domains that are responsible for mediating GA-induced degradation. The best characterised example being the *Arabidopsis gai-1* mutation, which confers a GA-insensitive dwarf phenotype (Koornneef *et al.*, 1985). This mutation results in a 17-amino-acid deletion from domain 1, also known as the DELLA domain, producing a constitutively active repressor of GA signaling that is not inactivated by GA (Peng *et al.*, 1997). Overexpression of genes containing analogous DELLA mutations in *RGA* (*rga-Δ17*) and *RGL1* (*rgl1Δ17*) resulted in GA-insensitive dwarf plants similar to *gai-1* (Dill *et al.*, 2001; Wen & Chang, 2002). Moreover, the *rga-Δ17* protein was not degraded in response to GA treatment, confirming the importance of the DELLA domain in GA-induced degradation (Dill *et al.*, 2001). In rice, a more detailed functional domain analysis of *SLR1* also highlighted the importance of domain 1 as well as that of the adjacent conserved domain 2 in this process (Itoh *et al.*, 2002). Based on these studies, it was originally proposed that the N-terminal domain of DELLA proteins is the domain responsible for regulating GA-induced degradation, whereas the C-terminal GRAS domain is the



functional domain. Recent studies now suggest that the GRAS domain may also play a regulatory role. A semi-dominant GA-insensitive dwarf mutant of *Brassica rapa* (*Brrgal-d*) with an amino acid substitution in the GRAS domain of the DELLA protein, BrRGA1, accumulated the protein to significantly higher levels than in the wild-type, and the mutant protein was insensitive to GA-induced degradation (Muangprom & Osborn, 2004; Muangprom *et al.*, 2005). Furthermore, it has now been shown that loss-of-function mutations in the GRAS domain of DELLA genes also prevent GA-induced degradation of the mutant proteins (Gubler *et al.*, 2002; Dill *et al.*, 2004). Thus, there appears to be no clearly defined domain within the DELLA proteins that regulates GA-induced degradation and it is conceivable that these domains have different roles in the degradation process.

#### 6.4.4 SCF<sup>SLY/GID</sup>-mediated degradation of DELLA proteins

Genetic screens for recessive GA-insensitive dwarf mutants in *Arabidopsis* and rice have led to the discovery of GA-signaling components that directly regulate DELLA protein stability in response to the GA-signal (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Dill *et al.*, 2004; Gomi *et al.*, 2004). Plants that are homozygous for the *Arabidopsis sleepy1* (*sly*) (Steber *et al.*, 1998) or rice *GA-insensitive dwarf 2* (*gid2*) (Sasaki *et al.*, 2003) alleles contain highly elevated levels of DELLA proteins and are unresponsive to GA (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004). Furthermore, genetic analyses demonstrate that DELLA loss-of-function mutations are epistatic to *sly1/gid2*. The *SLY1* and *GID2* genes have now been cloned and shown to encode F-box proteins (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004). The *Arabidopsis* genome contains a *SLY1* orthologue, designated *SNEEZY* (*SNE*), which has a related function since it is capable of complementing the *sly* mutant phenotype (Fu *et al.*, 2004; Strader *et al.*, 2004). The identification of loss-of-function mutations should help to address such a role for *SNE*.

Studies in yeast have demonstrated that F-box proteins are a core component of SCF E3 ubiquitin (Ub) ligases, which are multi-subunit complexes that recognise and poly-ubiquitinate target proteins destined for degradation by the 26S proteasome (Deshaies, 1999). The four main protein subunits of SCF E3 Ub ligases are the Skp1, cullin/Cdc53, Rbx1/Hrt1/Roc1 and F-box components (Zheng *et al.*, 2002). The F-box subunit provides the substrate specificity of the SCF E3 Ub ligase and is anchored to the Skp1-like subunit of the complex through an N-terminal F-box domain. The C-termini of F-box proteins are usually required for binding and targeting the substrate for ubiquitination, and often contain a conserved protein-protein interaction domain for this purpose (Gagne *et al.*, 2002). In many cases, recognition by the F-box protein requires the target protein to be post-translationally modified, most commonly by phosphorylation, although alternative forms of post-translational modification have also been shown to promote this association. The identity of *SLY1* and *GID2* as F-box proteins, together with the accumulation and GA insensitivity of DELLA proteins in the *sly1/gid2* mutants, is consistent with a role

in directly regulating GA-induced DELLA degradation as part of a SCF E3 Ub ligase complex. It has recently been confirmed that SLY1 and GID2 are components of the respective SCF<sup>SLY1</sup> and SCF<sup>GID2</sup> complexes. For example, in *Arabidopsis*, comprehensive yeast two hybrid assays were used to demonstrate that, of the 21 SKP1 homologues (ASK1 to ASK21; Gagne *et al.*, 2002), ASK1, ASK2, ASK3, ASK4, ASK11 and ASK13 interacted with both SLY1 and SNE (Fu *et al.*, 2004). It was subsequently confirmed *in planta* using co-immunoprecipitation that SLY1 interacts with both the ASK2 and AtCUL1 components of the SCF E3 Ub ligase. In rice, similar experiments have been used to confirm an interaction *in planta* between GID2 and the SCF components OsSkp15 and OsCUL1 (Gomi *et al.*, 2004).

Direct interaction between SLY1 and the *Arabidopsis* DELLA proteins RGA, GAI, RGL1 and RGL3 were demonstrated by yeast two-hybrid assays (Dill *et al.*, 2004; Fu *et al.*, 2004; Tyler *et al.*, 2004). Furthermore, a recombinant glutathione S-transferase (GST) SLY1 fusion protein was capable of interacting with endogenous RGA as well as *in vitro*-translated GAI and RGA protein in pull-down assays (Dill *et al.*, 2004; Fu *et al.*, 2004; Tyler *et al.*, 2004). Similar observations were obtained in rice, when it was demonstrated that recombinant GID2 interacts with SLR1 isolated from *gid2* plants (Gomi *et al.*, 2004). Interestingly, in *Arabidopsis*, a *SLY1* gain-of-function mutation, *sly1-d* (formerly *gar2-1*), results in increased GA signaling by reducing the levels of the DELLA protein in plants (Wilson & Somerville, 1995; Dill *et al.*, 2004; Fu *et al.*, 2004). This appears to be caused by an enhanced interaction between *sly1-d* and the DELLA proteins. An interaction between the DELLA proteins and SLY1/GID2 has yet to be demonstrated *in planta*, although it has been shown that SLY1/GID2 are nuclear localised, consistent with a role in GA-mediated DELLA degradation (Dill *et al.*, 2004; Gomi *et al.*, 2004).

In order to investigate how GA promotes recognition of DELLA proteins by the F-box subunit of the SCF<sup>SLY1/GID2</sup> E3 Ub ligase, several groups have focused on the potential role of DELLA phosphorylation in this process (Fu *et al.*, 2002; Sasaki *et al.*, 2003; Fu *et al.*, 2004; Gomi *et al.*, 2004; Hussain *et al.*, 2005). In rice, there was originally strong evidence that phosphorylation of SLR1 may promote its interaction with GID2, because SLR1 exists in the *gid2* background as both phosphorylated and non-phosphorylated forms, with only the phosphorylated form interacting with recombinant GID2 in pull-down assays (Sasaki *et al.*, 2003; Gomi *et al.*, 2004). However, the same group (Itoh *et al.*, 2005a) have shown recently that, GA-induced the accumulation of both phosphorylated and non-phosphorylated forms of SLR1 in *gid2* with similar kinetics. Furthermore, they showed that in wild-type cells treated with GA, both forms of SLR1 proteins were degraded with a similar half-life, and, in contrast to their previous results, both phosphorylated and non-phosphorylated forms of SLR1 interacted with GID2 in pull-down assays. Thus, it appears likely that the phosphorylation status of DELLA proteins is not GA responsive and does not target their degradation, at least in rice. This area clearly requires further study.

By analogy with studies of SCF E3 Ub ligase-mediated degradation in yeast and mammalian cells, it is expected that the interaction of DELLA proteins with the SCF<sup>SLY/GID2</sup> complexes would result in ubiquitination and subsequent degradation by the 26S proteasome. This appears to be the case, since GA treatment results in the accumulation of ubiquitinated forms of SLR1 in rice (Sasaki *et al.*, 2003). Furthermore, proteasome-specific protease inhibitors block the GA-induced degradation of some DELLA proteins (Fu *et al.*, 2002; Hussain *et al.*, 2005).

#### 6.4.5 *The role of GID1 in DELLA degradation*

The demonstration that GID1 is involved in the perception of bioactive GAs raises the question of its role in GA-induced degradation of SLR1. Ueguchi-Tanaka *et al.* (2005) provided direct evidence for such a role using both genetic and biochemical approaches. Firstly, they show that the *gid1/slr1* double mutant exhibits the *slr1* phenotype indicating that SLR1 is epistatic to GID1, and secondly, SLR1 protein levels are elevated in *gid1* compared to wild-type plants and are insensitive to GA-induced degradation. Furthermore, they demonstrated in yeast two-hybrid assays that GID1 interacts with SLR1 in a GA-dependent manner. Although it is necessary to confirm this interaction *in planta*, this finding provides an important link in the GA-signaling cascade. The next major step will be to understand whether this interaction plays a role in the recognition of SLR1 by the SCF<sup>GID2</sup> Ub E3 ligase. It is tempting to speculate that the N-terminal regulatory domain of the DELLA proteins may be necessary for the interaction between SLR1 and GID1, in which case the gain-of-function mutations in domains 1 and 2 of the DELLA proteins may prevent this interaction and render the proteins resistant to GA-induced degradation. Studies aimed at identifying the SLR1 domain responsible for interacting with GID1 should address this possibility.

#### 6.4.6 *Additional GA-signaling components*

The previous sections covering GA signaling describe a relatively simple signal transduction cascade composed of a few core components. It is clear from additional genetic and biochemical studies that this cascade is far more complex and involves other signaling components (Olszewski *et al.*, 2002). In the following sections the potential roles of several of the better characterised components will be discussed briefly.

##### 6.4.6.1 *A role for O-linked N-acetylglucosamine transferases in GA signaling*

*SPINDLY (SPY)* was the first GA-signaling gene to be cloned from *Arabidopsis* (Jacobsen *et al.*, 1996). The presence of the *spy* mutations will partially suppress all of the developmental defects caused by a block in GA biosynthesis, without increasing bioactive GA levels (Jacobsen & Olszewski, 1993; Silverstone *et al.*, 1997b; Silverstone *et al.*, 2001). The recessive nature of the *spy* mutations indicates

that *SPY* functions as a negative regulator of GA response (Jacobsen & Olszewski, 1993; Jacobsen *et al.*, 1996). Although these studies have illustrated the importance of *SPY* in GA signaling, its role currently remains unclear.

The *SPY* protein has homology with animal *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferases (OGTs), which catalyse the transfer of *N*-acetylglucosamine to serine or threonine residues of target proteins. This form of post-translational modification is a dynamic process that has a regulatory role akin to phosphorylation (Wells *et al.*, 2001). Although the function of OGTs is less well understood in plants than in animals, *in vitro* assays suggest that *SPY* has OGT activity (Thornton *et al.*, 1999), indicating that such activity may have a role in regulating plant development. In *Arabidopsis*, *SECRET AGENT* (*SEC*) encodes a second OGT with significant homology to *SPY* (Hartweck *et al.*, 2002). It is not clear whether *SEC* is involved in GA signaling because *sec* mutants do not display any obvious developmental defects. However, loss of both *SPY* and *SEC* activities in the *spy/sec* double mutant results in embryo abortion, indicating that OGT activity is necessary for embryogenesis (Hartweck *et al.*, 2002). This observation, together with the demonstration that *spy* mutants display developmental defects not observed in GA-response mutants (Swain *et al.*, 2001; Tseng *et al.*, 2004), indicate that OGTs are also involved in other aspects of plant growth and development. It seems likely that *SPY* modulates GA signaling by catalysing the *O*-GlcNAc modification of components of GA-response pathway. Considering the importance of DELLA proteins in this pathway, they are obvious candidates as *SPY* target proteins. It is conceivable that *O*-GlcNAc modification could regulate either the stability or activity of DELLA proteins. Biochemical studies aimed at investigating the *O*-GlcNAc status of DELLA proteins will be necessary to test this hypothesis.

#### 6.4.6.2 *DWARF1* and *PHOR1*, possible positive regulators of GA signaling

Biochemical and genetic evidence indicating that the  $G\alpha$  subunits of heterotrimeric G proteins acts as a positive regulator of GA response has prompted an interest in the potential role of G proteins in GA signaling (Jones *et al.*, 1998; Ashikari *et al.*, 1999; Fujisawa *et al.*, 1999; Ueguchi-Tanaka *et al.*, 2000). Such a role is most apparent in rice, for which the *d1* mutant, which exhibits a GA-insensitive dwarf phenotype, has a defective  $G\alpha$  protein (Ashikari *et al.*, 1999; Fujisawa *et al.*, 1999). By analogy with heterotrimeric G proteins in mammalian systems, it is possible that a G protein coupled receptor acts as a membrane-bound GA receptor and transduces the signal through its interaction with the  $G\alpha$  (Ashikari *et al.*, 1999; Lovegrove & Hooley, 2000). However, there are now several lines of evidence which argue against this model: firstly, the identification of *GID1* as a soluble GA receptor that mediates and is required for all GA responses in rice is not clearly compatible with the heterotrimeric G protein model. Secondly, in rice and *Arabidopsis* a single gene encodes the  $G\alpha$  subunit, but loss-of-function mutations in these genes result in reduced GA responsiveness rather than a complete loss (Ueguchi-Tanaka *et al.*, 2000; Ullah *et al.*, 2001; 2002). Thirdly,  $G\alpha$  knock-out mutants also demonstrate

defects in several additional signaling pathways that are not commonly observed in well-characterised GA-response mutants (Ullah *et al.*, 2001; Suharsono *et al.*, 2002; Ullah *et al.*, 2002). Therefore, it is currently not clear whether the role of the  $G\alpha$  proteins is to regulate downstream GA responses directly or to modify sensitivity to the hormone. The identification and characterisation of downstream targets of the  $G\alpha$  subunits should provide further insights into their roles.

The photoperiodic control of tuberisation in *Solanum tuberosum* (potato) is mediated, in part, by GAs, which inhibit tuberisation (Amador *et al.*, 2001a). A study by Amador *et al.* (2001b) aimed at understanding the photoperiodic induction of tuberisation resulted in the identification of *PHOR1* (*PH*Otoperiod-*R*esponsive *1*), which encodes an arm repeat protein that may function as a positive regulator of GA signaling. Transgenic potato plants with reduced levels of PHOR1 are semi-dwarf and have reduced GA responses. Gibberellins promoted the relocalisation of a PHOR1–GFP fusion protein from the cytoplasm to the nucleus, in transiently transformed tobacco BY2 cells. The PHOR1 protein contains a U-box (UFD2 homology) domain, which is common to a class of E3 ubiquitin ligases (Koegl *et al.*, 1999), raising the possibility that it regulates GA signaling by promoting the degradation of target proteins through the 26S proteasome. In *Arabidopsis*, there are three genes (HIM1, 2 and 3) that share substantial homology to PHOR1. Characterisation of these should provide important clues to the role of PHOR1-related genes in GA signaling.

## 6.5 Downstream transcriptional events induced by GAs

The identification of the genes that are the final targets of the GA-signaling pathway is clearly essential for understanding GA function. Studies of the GA-induced secretion of  $\alpha$ -amylase and other hydrolytic enzymes from cereal aleurone cells have been extremely important in understanding how GA regulates gene transcription. The wealth of information provided by the aleurone system is impossible to cover comprehensively here and the reader is referred to recent excellent reviews of the subject (Lovegrove & Hooley, 2000; Olszewski *et al.*, 2002; Sun & Gubler, 2004). Much of the recent advances in this field have focused on the involvement of GAMYB transcription factors and this is described in the following section.

Although stimulation of stem extension is one of the classical functions of GAs, this process has been less well studied than the cereal aleurone in terms of the gene targets. Gibberellins promote stem extension by stimulating cell elongation and/or cell division. There are reports suggesting that GA-induced cell elongation is mediated, in part, through the action of xyloglucan endotransglycosylase/hydrolases (XETs) and expansins, which enable loosening of the cell wall (Nishitani, 1997; Cosgrove, 2000). Transcript analysis demonstrating GA-induced upregulation of expansin and XET genes has been described for several plants, including *Arabidopsis*

and rice (Smith *et al.*, 1996; Xu *et al.*, 1996; Cho & Kende, 1997; Lee & Kende, 2002; Ogawa *et al.*, 2003). In deepwater rice, which undergoes rapid stem elongation following submergence, GA promotes the expression of *OsEXP4* in internodes within 30 min of treatment, indicating that this is an early GA response gene (Cho & Kende, 1997). Interestingly, promoter analysis has identified the presence of GA Response Elements (GARE) in some of the rice expansin genes (Lee *et al.*, 2001). It will be interesting to see whether the mechanisms of GA-induced expression of *OsEXP4* are similar to those regulating  $\alpha$ -amylase. In addition to promoting cell expansion, the submergence of deepwater rice promotes stem elongation through an increase in cell division in the intercalary meristem. This cell division activity is induced by GA at the G1 to S phase transition (Lorbiecke & Sauter, 1998). Furthermore, GA regulates G2/M phase progression by inducing the expression of genes encoding cyclin-dependent protein kinases, including *cycA1;1* and *cdc2Os-3* (Fabian *et al.*, 2000).

The availability of DNA microarrays has provided an important tool to dissect the transcriptional changes that promote GA-responsive growth. The potential of this approach has recently been highlighted by the studies of Ogawa *et al.* (2003), who used gene profiling on Affymetrix arrays to investigate the action of GAs in *Arabidopsis* seed germination. Treatment of *gal-3* seeds with GA for 12 h resulted in 230 GA-upregulated and 127 GA-downregulated genes, with a greater than four-fold change in expression. In support of their role in GA-induced germination, the expression profiles of most of these genes were found to change in a similar fashion during the germination of wild-type seeds. Somewhat surprisingly, changes in expression of many of these genes preceded the rise in endogenous GA levels, perhaps indicating response to GA present in the seed before imbibition. The identities of the GA-responsive genes found in this study is providing new insights into the mechanisms that control GA-mediated germination. For example, many genes that are implicated in cell elongation and cell division were identified, including those encoding for expansins, XETs, aquaporins, a D-type cyclin and a replication protein A. Interestingly, an ABA-responsive element was overrepresented in the promoters of the GA-downregulated genes, suggesting that it may be also a GARE. Future studies aimed at understanding both the role and regulation of these GA-responsive genes will undoubtedly enhance our understanding of how GAs regulate germination and other developmental processes.

### 6.5.1 *GAMYBs*

In barley (*Hordeum vulgare*) activation of  $\alpha$ -amylase expression is induced by binding of GAMYB to the GARE of the promoter (Gubler *et al.*, 1995; Gubler *et al.*, 1999). It has been demonstrated that GA response mediated through GAMYB is dependent on the DELLA proteins SLN1 and SLR1, in barley and rice, respectively (Ikeda *et al.*, 2001; Fu *et al.*, 2002; Gubler *et al.*, 2002), in which the DELLA proteins act as negative regulators of GAMYB-mediated gene expression.



The expression of *GAMYB* in tissues other than aleurone in barley, rice, *Lolium*, wheat and *Arabidopsis* indicates that it functions in other GA-dependent processes. Furthermore, the recent characterisation of *GAMYB* knock-out mutants in rice and *Arabidopsis* has provided more conclusive evidence for such roles. In rice, which contains a single *OsGAMYB* gene, mutations in this gene abolished  $\alpha$ -amylase expression in response to GA treatment (Kaneko *et al.*, 2004). Although the *Osgamyb* plants did not have any obvious defects in vegetative development, or in the timing of floral transition, they did display abnormalities in floral organ development. For example, there were often dramatic defects in pollen development, resulting in male sterility. Interestingly, there was a large variation in the severity of the phenotype, which Kaneko *et al.* (2004) suggested was caused by the environmental conditions. The situation in *Arabidopsis* is complicated by the presence of at least three putative *GAMYB* genes: *AtMYB33*, *AtMYB65* and *AtMYB101* (Gocal *et al.*, 2001; Achard *et al.*, 2004; Millar & Gubler, 2005). The findings that each of these three genes is capable of substituting for Hv*GAMYB* in transactivating the barley  $\alpha$ -amylase promoter provides support for their roles in GA signaling (Gocal *et al.*, 2001). Single knock-outs of *Atmyb33* and *Atmyb65* are indistinguishable from wild-type plants, indicating functional redundancy (Millar & Gubler, 2005), while the *Atmyb33/Atmyb65* double knock-out is male sterile due to defects in pollen development. This phenotype is consistent with the expression of *AtMYB33*, which is predominantly localised to the anthers in floral tissues. As in rice, the penetrance of the *Atmyb33/Atmyb65* mutant phenotype is variable and subject to environmental control, low light intensity and high temperatures dramatically enhancing the male sterility of the mutant plants.

Three microRNAs (miRNAs; miR159a, b and c), recently identified in *Arabidopsis*, have high complementarity to the coding region of the three *GAMYB* genes, in addition to two other *AtMYBs* (Rhoades *et al.*, 2002). It was demonstrated recently that miR159 directs the cleavage of *AtMYB33* and *AtMYB65* transcripts (Palatnik *et al.*, 2003; Achard *et al.*, 2004). However, if this cleavage is prevented by expression of a mutant *AtMYB33* lacking the miR159 complementary sequence, the resulting transgenic plants display pleiotrophic developmental defects (Palatnik *et al.*, 2003; Millar & Gubler, 2005). These severe abnormalities are in contrast to the specific floral defects observed in the *myb33/myb65* double mutant. The observation that expression of the mutant *myb33* is increased in additional tissues outside of the anthers (Millar & Gubler, 2005) provides an explanation for this and supports a role for miR159 in restricting the expression pattern of *AtMYB33*.

Although the function of *GAMYBs* in regulating GA responses in aleurone cells is well defined, it is not clear whether their role in anther development is directly related to GA signaling. Continuous exogenous treatment of *Arabidopsis* plants with GA does increase *MYB33* transcript levels, but this appears to be independent of the GA signaling status of the plants (Achard *et al.*, 2004). In contrast, the levels of miR159 are induced by GA via opposition of DELLA function, suggesting that miR159 may function as a GA-response component by regulating *GAMYB* activity. It remains to be determined why alterations in miR159 levels found in GA-biosynthetic/signaling



mutants do not translate into changes in MYB33 transcript levels. It is conceivable that miR159 regulates MYB33 activity through translational control.

### 6.5.2 Homoeostatic regulation of GA metabolism

It is well documented that plants maintain the levels of endogenous bioactive GAs through the processes of feedback and feedforward regulation of GA metabolism (reviewed by Hedden & Phillips, 2000). Biochemical studies and transcript profiling experiments have demonstrated that the metabolic steps catalysed by the 2-ODDs are predominantly involved in this homoeostatic regulatory mechanism (Chiang *et al.*, 1995; Phillips *et al.*, 1995; Xu *et al.*, 1995; Thomas *et al.*, 1999). For example, in *Arabidopsis*, expression of the GA-biosynthetic genes, *AtGA3ox1* (formerly *GA4*) and *AtGA20ox1* (formerly *GA5*), are elevated in a GA-deficient background and then subsequently reduced following treatment of the plants with bioactive GAs (Chiang *et al.*, 1995; Phillips *et al.*, 1995). In contrast, expression of the GA-inactivating enzymes, *AtGA2ox1/2*, are elevated when GA-deficient plants are treated with bioactive GAs (Thomas *et al.*, 1999).

The molecular mechanisms that underlie this transcriptional regulation of the 2-ODDs are currently unknown, although, it is apparent that the GA-signal transduction cascade is necessary for this response. This is illustrated by the findings that feedback regulation is perturbed in GA-response mutants (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Dill & Sun, 2001; McGinnis *et al.*, 2003) and the direct correlation between the levels of functional DELLA proteins and the expression of the GA-metabolic genes. For example, in the *gal-3* mutant, DELLA protein and *AtGA3ox1* transcript levels are elevated and then rapidly reduced by bioactive GA treatment (Silverstone *et al.*, 2001), while the DELLA loss-of-function mutant, *gal-3/gai-16/rga-24*, has reduced levels of *AtGA3ox1* transcripts compared to *gal-3* and these are unaffected by GA treatment (Dill & Sun, 2001; King, K.E. *et al.*, 2001). Furthermore, DELLA gain-of-function mutations, such as *gai-1* and *rga-Δ17*, result in constitutively high expression levels of *AtGA3ox1* which are unaffected by GA treatment (Dill *et al.*, 2001). These observations support a role for DELLA proteins as positive regulators of the 2-ODD genes that are under feedback control.

Feedback regulation of 2-ODD genes are some of the fastest GA responses. Decreases in *AtGA3ox1* and *AtGA20ox1* transcript levels in *gal-3* are observed within 30 min of treatment with GA (Ogawa *et al.*, 2003). This raises the possibility that DELLA proteins regulate transcription of these genes directly, despite the absence of a conserved DNA-binding domain within the DELLA protein (Peng *et al.*, 1997; Silverstone *et al.*, 1998). It is, indeed, possible that DELLA proteins act as part of a transcriptional complex. The finding that cycloheximide blocks the feedback regulation of *AtGA20ox1* expression indicates the requirement for protein synthesis in this response (Bouquín *et al.*, 2001), although, this would not necessarily require an earlier round of transcription. It is clear that one of the next major challenges in this field is to understand the mechanism by which DELLA proteins regulate transcript levels of GA-response genes.

## 6.6 Sites of GA signaling

A major challenge in our attempts to understand how GAs regulate the many different facets of plant growth and development is to determine the precise sites of GA metabolism and action. Biochemical studies have proved highly successful in demonstrating that in some cases, most notably in germinating cereal grains, GA acts as a paracrine signal, biosynthesis and response occurring in separate locations. In contrast, the observations that GA biosynthesis is feedback regulated via the signaling pathway imply that they occur in the same cells in many tissues. In recent years a number of studies have taken the opportunity provided by the availability of genes encoding GA biosynthetic and signaling components to determine their precise cellular localisation. Several examples are discussed in the following sections.

### 6.6.1 Germinating seeds

In germinating cereal seeds, GAs, which are synthesised in the embryo, promote  $\alpha$ -amylase expression in the aleurone cells (Jones & Jacobsen, 1991). In order to obtain more information on the relationship between these distinct processes and how they control germination, Kaneko *et al.* (2003) investigated the cellular localisation of transcripts encoding GA-biosynthetic enzymes and signaling components in germinating rice seeds. To determine where bioactive GAs are produced, the expression patterns of four biosynthetic genes, *OsGA20ox1*, *OsGA20ox2*, *OsGA3ox1* and *OsGA3ox2* were monitored. Although all of these transcripts were detected in the embryo, there were two distinct expression patterns. Transcripts of *OsGA3ox1* and *OsGA20ox1* were present exclusively in the epithelium of the scutellum, whereas *OsGA3ox2* and *OsGA20ox2* were expressed also in the developing shoot. Interestingly, expression of *OsGA3ox2*, but not that of *OsGA3ox1*, in the epithelium appears to be necessary for producing GAs for induction of  $\alpha$ -amylase expression (Kaneko *et al.*, 2002). *SLR1*, which encodes a component of GA-signal transduction, was expressed in essentially the same tissues as *OsGA3ox2* and *OsGA20ox2* within the embryo, indicating that bioactive GAs are being produced at their site of action. However, *SLR1*, but not the GA-biosynthetic genes, was also expressed in the aleurone, confirming that this tissue is dependent on the import of GAs for  $\alpha$ -amylase expression (Fincher, 1989).

Similar studies have been conducted in *Arabidopsis* seed, which require *de novo* GA biosynthesis for germination (Yamaguchi *et al.*, 2001; Ogawa *et al.*, 2003). Imbibition of *Arabidopsis* seeds results in the upregulation of expression of multiple genes encoding GA-biosynthetic enzymes (see Section 6.5). Results from studies using *in situ* hybridisation and analysis of promoter:GUS reporter lines suggested that GA biosynthesis occurs in two separate locations within the embryo axis; the early steps occurring in the provasculature and the later steps in the cortex and endodermis, where bioactive GAs promote cell expansion (Yamaguchi *et al.*, 2001). This requires intercellular transport of GA precursors, possibly *ent*-kaurene.

The transcriptional profiling experiments described in Section 6.5 have uncovered many response genes that are regulated by GA in the germinating *Arabidopsis* seed (Ogawa *et al.*, 2003). The identity of these genes has provided an important opportunity to compare the sites of GA production with those where downstream transcriptional events are occurring (Ogawa *et al.*, 2003). Analysis of the GA-induced expression profiles of three strongly GA-responsive genes by *in situ* hybridisation indicated that their GA-responsive expression was not restricted to the cortex/endodermal cells where bioactive GAs were produced. This finding supports the movement of bioactive GAs (or a GA-induced signal) within the germinating *Arabidopsis* seeds.

### 6.6.2 Stems

One of the classical roles of GAs is to promote stem elongation. Although this discovery was made over 50 years ago, the sites of GA metabolism and action in the stem have not been clearly defined. One of the first studies on the site of GA biosynthesis in stem tissues was by Aach *et al.* (1997), who showed that *ent*-kaurene synthesis occurred in pro-plastids within the wheat intercalary meristem. More recently, Kaneko *et al.* (2003) determined the expression patterns of the GA-biosynthetic genes *OsGA20ox2* and *OsGA3ox2* and signaling component *SLR1* in elongating rice stems. On the basis of promoter::GUS reporter lines, they found that expression patterns of these genes were similar and occurred predominantly in the nodes and in the cell division and elongation zones, but not in fully elongated cells of the internodes. These observations indicate that GA biosynthesis occurs at the sites of its action in the rice stem, in regions that are undergoing cell division and elongation. Similarly, in wheat, the GA-biosynthetic gene *TaGA3ox2* is expressed in the nodes, internodes and the ear of the elongating stem, with highest expression levels in the lower part of the internode, where elongation is occurring (Appleford *et al.*, 2006). However, on the basis of Northern blot analysis, the GA 20-oxidase gene *TaGA20ox1* was shown to be much more highly expressed in the node than in the internode. This might indicate movement of an intermediate, presumably GA<sub>20</sub>, from the intercalary meristem to the elongation zone. It is interesting to note that expression of the wheat orthologue of *OsGA20ox2* was not detected in the elongating stem, suggesting that different GA 20-oxidase genes may control stem elongation in rice and wheat.

### 6.6.3 Flower initiation and development

Gibberellins have been implicated in promotion of flowering in several species (reviewed by Pharis & King, 1985; see also Chapter 10). The temperate grass, *L. temulentum*, which flowers in response to a single LD exposure, has provided an useful model system for studying GA-induced flowering (King & Evans, 2003). Perception of the LD photoperiod occurs in the leaf and a signal is exported to the apex within 24 h. There is now compelling evidence supporting the role of GAs as

this signal. Initially, it was demonstrated that the application of specific GAs can substitute for the LD exposure in promoting flowering in *L. temulentum* (Evans *et al.*, 1990; King, R.W. *et al.*, 2001; King *et al.*, 2003). Interestingly, GA<sub>5</sub> and GA<sub>6</sub> are more effective in floral induction than GA<sub>1</sub> and GA<sub>4</sub>, which are the most important growth-active GAs. Furthermore, GA<sub>5</sub> and GA<sub>6</sub> levels increase in the leaves following a single LD exposure and a subsequent two-fold rise in the apex is observed 24 h later (King, R.W. *et al.*, 2001; King *et al.*, 2003). Although GA<sub>1</sub> and GA<sub>4</sub> are detectable in leaves prior to floral induction, they remain undetectable in the vegetative apex (Gocal *et al.*, 1999). It has been proposed that GA<sub>1</sub> and GA<sub>4</sub> are excluded from the vegetative apex by the action of a GA 2-oxidase, which deactivates these compounds, but is ineffective against GA<sub>5</sub> and GA<sub>6</sub> (King & Evans, 2003). Support for this hypothesis is provided by studies in rice demonstrating expression of *OsGA2ox1* in a ring surrounding the vegetative apex (Sakamoto *et al.*, 2001b). Expression of *OsGA2ox1* is substantially reduced following the phase transition to an inflorescence meristem. If a similar situation occurs in *L. temulentum* it could also provide an explanation for the observed increases in GA<sub>1</sub> and GA<sub>4</sub> levels at the apex several days after floral induction (King, R.W. *et al.*, 2001). It has been suggested that these increases are necessary for promoting inflorescence development. Further characterisation of GA metabolic and signaling components in *L. temulentum* in combination with reverse genetics approaches in model plants should help to uncover the role of GAs in mediating floral induction.

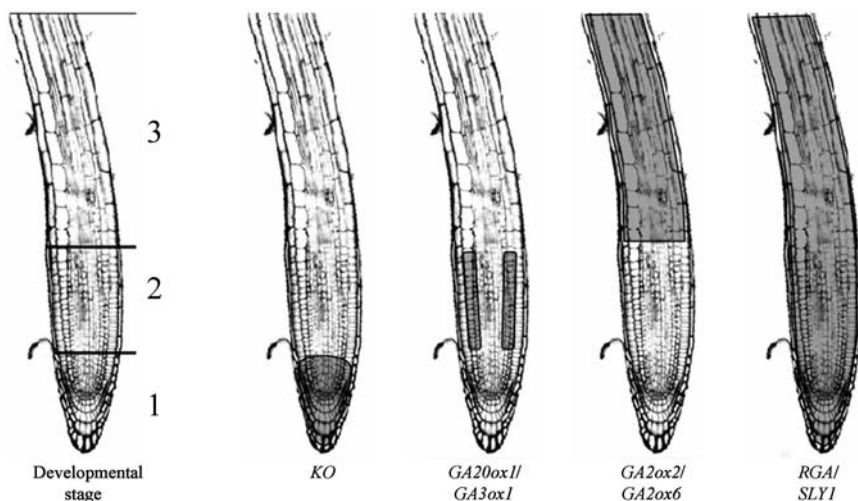
In addition to their role in promoting flowering, GAs are also involved in promoting development of the floral organs (see Chapter 10). In many plants the predominant site of GA biosynthesis appears to be the anthers. It has been proposed that GAs produced in the anthers is necessary for the development of other floral organs (Weiss *et al.*, 1995). In rice, the study of the sites of GA biosynthesis and signaling by Kaneko *et al.* (2003) provided support for this model. They demonstrated that the biosynthetic genes *OsGA3ox2* and *OsGA20ox2* were predominantly expressed in the stamen primordia and developed anthers, whereas expression of the signaling component, *SLR1*, was found in additional floral organs. Although *OsGA3ox2* and *OsGA20ox2* play a major role in producing bioactive GAs in many tissues of the rice plant, *OsGA3ox1* and *OsGA20ox1* appear to have more tissue-specific roles that were also noted during germination of the seed. In flowers, both of these genes are specifically expressed in tapetum cells of the anthers.

#### 6.6.4 *The Arabidopsis root*

The examples described in the previous three sections illustrate a gene-by-gene approach to localisation of GA-signaling elements. The availability of DNA oligonucleotide microarrays provides a high-throughput method to localise the expression of all of these elements by obtaining global gene transcript profiles from individual cell types within an organ. The potential of this approach has been demonstrated in an elegant study by Birnbaum *et al.* (2003) in which they determined gene expression profiles for specific cell types and developmental stages

within the *Arabidopsis* root. This was achieved using transgenic lines expressing GFP in specific cell populations. Protoplasts prepared from these lines were used to isolate the specific cell types using fluorescence-activated cell sorting. A combination of the expression profiles from the cell types and distinct developmental stages were used to create a global map of gene expression within 15 zones of the root tip.

Although the function of GAs in regulating *Arabidopsis* root growth is currently poorly understood, the presence of transcripts encoding GA-metabolic enzymes and signaling components in this tissue supports such a role (Thomas *et al.*, 1999; Silverstone *et al.*, 2001; Wang *et al.*, 2004). Further evidence is provided by the recent findings that the *gal-3* mutants have shorter primary roots than wild-type plants, which are fully rescued by exogenous GA treatment (Fu & Harberd, 2003). The DELLA proteins, RGA and GAL, appear to function as the predominant repressors of GA-mediated root growth in *Arabidopsis* (Fu & Harberd, 2003). The global map of gene expression in the *Arabidopsis* root now provides an important opportunity to determine more precisely the cellular localisation of GA-signaling components (Fig. 6.3). The GA-signal transduction components *RGA* and *SLY1* are expressed constitutively throughout the root tip, suggesting that the majority of these cells are capable of responding to bioactive GAs. One intriguing aspect of the root expression data is the apparent localisation of the genes of GA metabolism.



**Figure 6.3** Localised expression of GA metabolic and signaling genes in *Arabidopsis* roots. Reprinted from Birnbaum *et al.* (2003). Numbers indicate developmental stages profiled, which were dissected with the use of the following landmarks as upper borders: stage 1, where the root tip reached its full diameter (about 0.15 mm from the root tip); stage 2, where cells transition from being optically dense to a more transparent appearance as they begin longitudinal expansion (about 0.30 mm from the root tip) and stage 3, where root hairs were fully elongated (about 0.45 to 2 mm from the root tip). Expression profiles (shaded regions) were determined for: stele, endodermis, endodermis plus cortex, epidermal atrichoblast cells and lateral root cap. Expression of *GA20ox1* and *GA3ox1* was detected predominantly in endodermis plus cortex at stage 2.

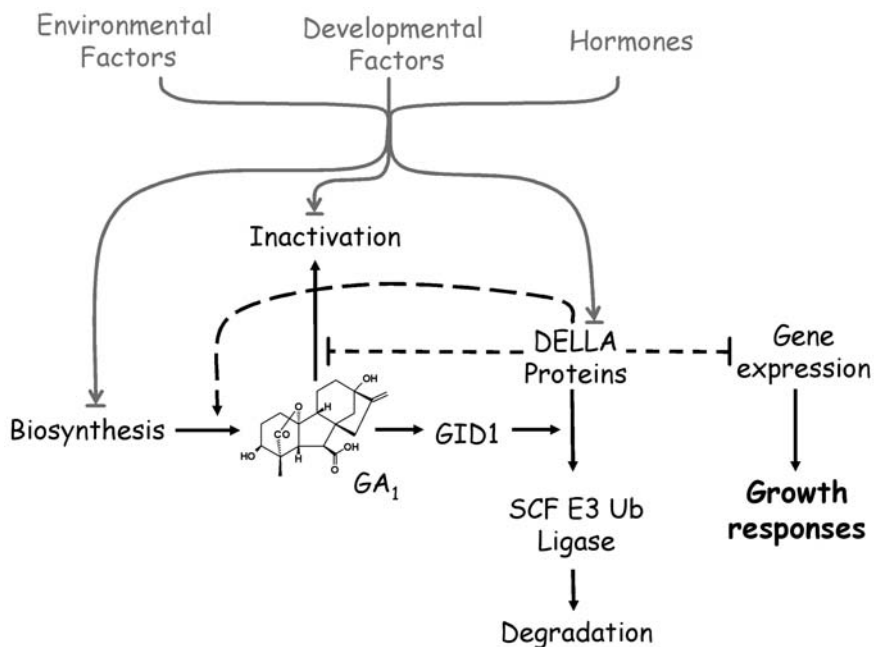
Expression of the early biosynthetic gene, *KO*, occurs predominantly in the root tip, developmental stage 1 (Fig. 6.3). The later biosynthetic genes, including *GA20ox1* and *GA3ox1* are expressed at a later developmental stage (stage 2). It is also interesting to note that *GA20ox1* and *GA3ox1* display a tissue-specific expression pattern, with their transcripts accumulating predominantly in endodermal and cortical cells. In contrast to the biosynthetic genes, expression of the GA-inactivating enzymes, *AtGA2ox2* and *AtGA2ox6*, are predominantly in developmental stage 3, where elongation of the root hairs is complete. One possible explanation for this expression profile of GA-metabolic components is that biosynthesis of bioactive GAs is required to promote longitudinal expansion within the root elongation zone. When elongation is complete, the bioactive GAs are inactivated reducing GA-promoted cell expansion. The characterisation of mutants containing loss-of-function mutations in GA-metabolic genes will provide a useful resource to test this hypothesis.

## 6.7 Conclusions

Our knowledge of GA signaling has advanced impressively. A simplified model of this pathway, including biosynthesis and signal transduction, is shown in Fig. 6.4, which indicates the relationship between signal transduction and metabolism via the feedback/feedforward mechanism. The recent discovery of a soluble GA receptor has closed several gaps in the model by revealing the signal transduction pathway to be much shorter than anticipated, with GA involved surprisingly directly with the turnover of DELLA proteins. Of course, there is still much to learn, including the relationship between the receptor and the F-box protein in targeting DELLA proteins for degradation. The possible contribution of membrane receptors, for which there is considerable indirect evidence, remains unclear, as does the immediate targets for the DELLA proteins and the mechanism by which they regulate transcription. A major challenge is the identification of genes that are regulated by GA signaling, directly or indirectly, and to determine how their activity translates into the physiological consequences of GA action.

Knowledge of the identity of most of the components of the GA-signaling pathway is enabling progress to be made in our understanding of how this process is regulated by developmental and environmental cues. Research in this area has focused particularly on the regulation of expression of the GA-biosynthetic and deactivation genes, the results suggesting that control of GA content is of major importance in many plant developmental processes. However, there has been so far little consideration of the involvement of enzyme stability in controlling GA metabolism, although protein degradation plays a pivotal role in GA-signal transduction. This subject clearly deserves more attention despite the technical difficulties associated with measuring the very low abundance of GA-metabolic enzymes in most tissues. An associated problem, which is receiving attention, is determining the cellular sites of GA biosynthesis and signal transduction. Progress in this area will be facilitated by cell specific transcript profiling using methods such as





**Figure 6.4** *Regulation of GA-responsive growth and development.* In the absence of bioactive GAs, DELLA proteins repress GA-dependent growth by regulating the expression of primary GA-response genes. This repression is relieved when bioactive GA levels are raised in response to environmental, developmental and hormonal signals, either by increasing GA biosynthesis or reducing its inactivation (or a combination of both). The increase in GA levels leads to GID1-mediated ubiquitination of the DELLA proteins via an SCF E3 Ub ligase and subsequent degradation by the 26S proteasome (see Fig. 6.2). The resulting decrease in DELLA protein levels promote changes in the expression of primary GA-response genes, which in turn promotes GA-dependent growth and development. These responses include the homeostatic regulation of GA metabolism. In this case, GA signaling rapidly downregulates the expression of GA-biosynthetic genes whereas GA-inactivating genes are upregulated. It is evident that developmental, environmental and hormonal signals are also responsible for regulating the GA-signaling cascade. This is most clearly illustrated for the DELLA components, although it is likely that other signaling components are also regulated by these signals.

laser capture micro-dissection and fluorescence-activated cell sorting, whereas the increasing sensitivity of mass spectrometers will allow ever smaller samples to be analysed. The available evidence, based mainly on transcript distribution or patterns of reporter gene expression, indicates that GAs may be produced and function in the same cells (i.e. they act as autocrine signals). The existence of a soluble, nuclear-localised receptor and of feedback regulation of GA biosynthesis is consistent with this scenario. However, there are also clear indications of paracrine function in specific cases, such as in the cereal grain. The regulation of GA signaling via interactions with other hormones is proving an important mechanism for integrating hormone signals. In particular, the accumulating evidence that GA



biosynthesis and signal transduction are both stimulated by auxin suggests that GAs may mediate some of the responses of this long-distance messenger locally. Future research will provide further insight into these issues and build on the firm base that has now been established.

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## 7 Oxylipins: biosynthesis, signal transduction and action

Claus Wasternack

### 7.1 Introduction

Plants have to adapt to the environment due to their sessile lifestyle. Consequently, constant monitoring of abiotic factors of the environment, such as light, oxygen, water, osmotic pressure, salt, temperature, nutrients (e.g. glucose, nitrogen and phosphate), gravity, wind, touch or chemicals have to be performed by independent perception and transduction pathways. In many cases, however, factors reach unfavourable levels corresponding to stress. Furthermore, biotic interactions between plants and pathogenic or symbiotic microorganisms and herbivores or between different plants may occur. Due to the high number of herbivorous insects and pathogenic microorganisms, there are numerous complex interactions (Fig. 7.1).

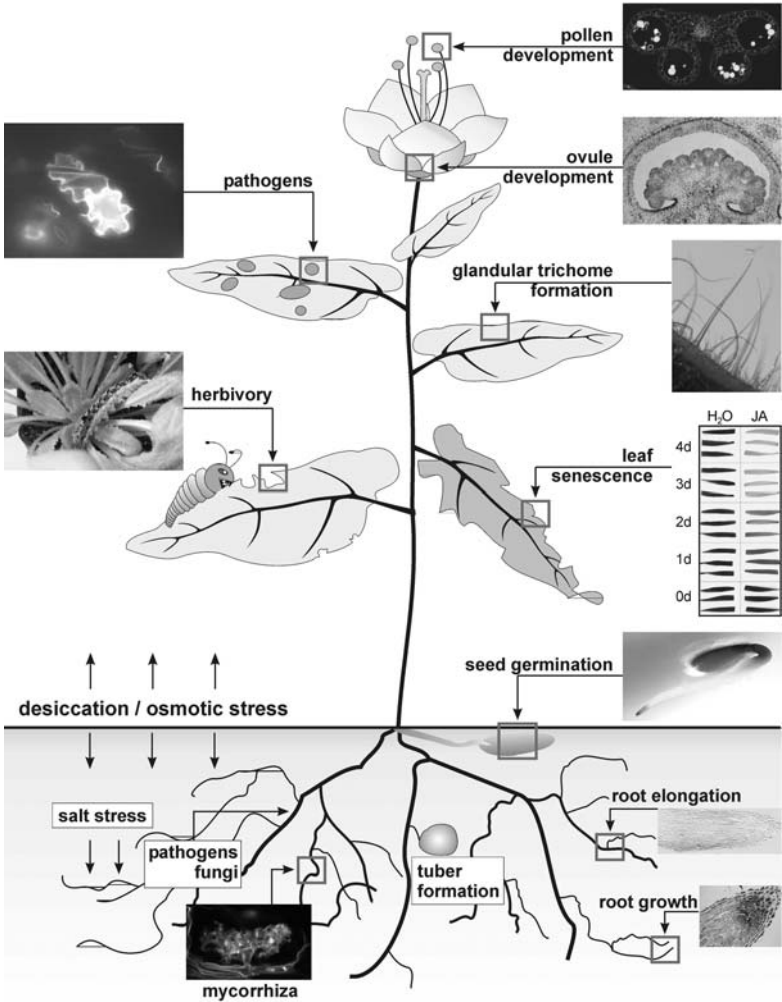
In the past 15 years, jasmonic acid (JA) and metabolites, such as its methyl ester (MeJA) or amino acid conjugates of JA, all of them commonly named jasmonates were recognized as important signals in plant responses to biotic and abiotic stress. Jasmonates originate from the common precursors 12-oxophytodienoic acid (OPDA) and dinor-OPDA (dnOPDA), which are collectively called octadecanoids. These JA precursors are formed within the lipoxygenase (LOX) pathway, which is initiated by oxygenation of free or esterified polyunsaturated fatty acids (PUFAs) leading to many different products collectively named oxylipins. More recently, octadecanoids and some other oxylipins were recognized to be JA-independent signals in plant stress responses.

Beside analysis of biosynthesis of all these oxylipins, increasing interest is given to their perception and signal transduction. Here, transgenic approaches and analyses of mutants led to fundamental knowledge on JA signaling, including a remarkable cross-talk with signaling pathways of other plant signals (hormones), such as salicylate, ethylene or abscisic acid. In *Arabidopsis thaliana*, a common phenotype of JA deficiency and JA insensitivity is male sterility. Since identification of the first JA-insensitive male sterile mutant in 1994 (Feys *et al.*, 1994) increasing knowledge has accumulated on the role of jasmonates and other oxylipins in distinct developmental processes. These data led to deeper insights into various developmental processes, such as root growth or senescence for which JA was already recognized as a stimulatory or inhibitory effector by physiological experiments in the 1980s (Fig. 7.1).

This chapter will cover recent aspects of biosynthesis, signal transduction and action of jasmonates and other oxylipins. As usually in a field of high interest, constantly

biotic and abiotic stress

development



**Figure 7.1** Scheme showing biotic and abiotic stress responses and developmental processes that are known to be jasmonate dependent. The following processes are highlighted: pathogen attack (*Phytophthora infestans*) on potato leaf, autofluorescence of an attacked cell (photo: J. Landtag); herbivory (*Spodoptera littoralis*) on *Arabidopsis* (photo: B. Hause); mycorrhiza (*Glomus intraradices*) on roots of *Medicago truncatula* (photo: B. Hause); pollen development (cross-section of an *Arabidopsis* anther, photo: B. Hause); ovule development (cross-section of a tomato flower bud with immunolocalization of AOC, photo: B. Hause); glandular trichome development (trichomes of tomato leaf stalk, photo: H. Paetzold); leaf senescence (barley leaf segments floated on water or JA, photo B. Hause); seed germination (tomato seedling showing AOC promoter activity by GUS staining, photo: I. Stenzel); root elongation (primary tomato root with AOC promoter activity, photo: B. Hause); root growth (tomato root tip with immunostaining for AOC, photo: B. Hause).

appearing reviews are available on specific aspects (e.g. Walling, 2000; Blée, 2002; Feussner & Wasternack, 2002; Gatehouse, 2002; Wasternack & Hause, 2002; Farmer *et al.*, 2003; Halitschke & Baldwin, 2004; Howe, 2004; La Camara *et al.*, 2004; Mithöfer *et al.*, 2004; Pauw & Memelink, 2004; Pozo *et al.*, 2004; Schaller *et al.*, 2004; Lorenzo & Solano, 2005; Rosahl & Feussner, 2005; Schilmiller & Howe, 2005). Here, I review recent progress in biosynthesis, signal transduction and role of oxylipins. (I apologize for references not cited due to space limitation.)

## 7.2 $\alpha$ -Dioxygenase, phytoprostanes and electrophile compounds

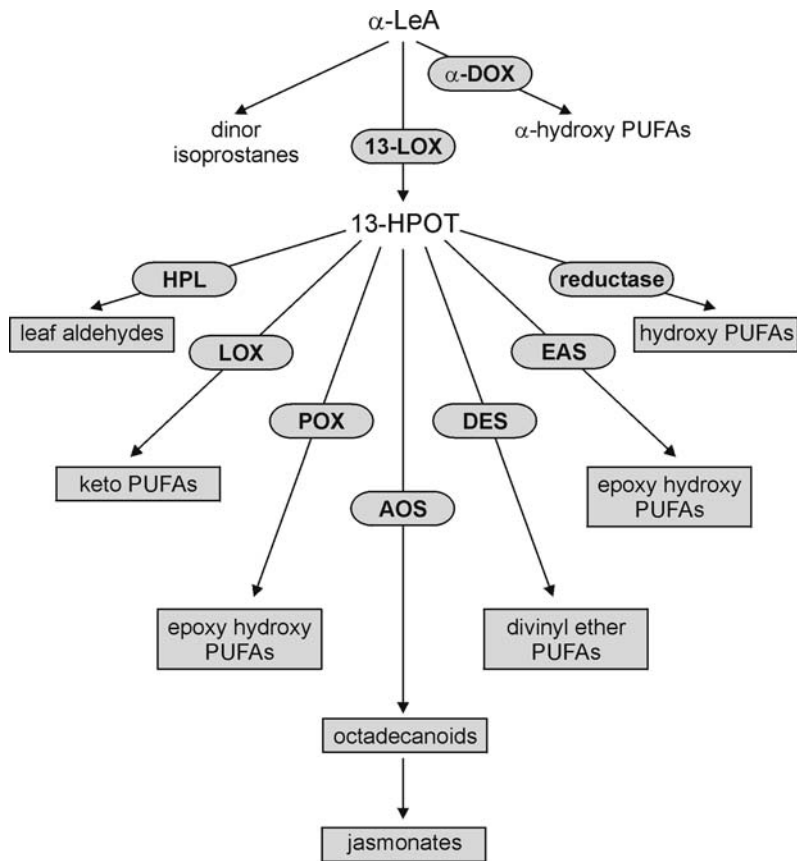
### 7.2.1 $\alpha$ -Dioxygenase

In saturated and unsaturated fatty acids molecular oxygen can be inserted at the C-2 by an  $\alpha$ -dioxygenases ( $\alpha$ -DOX), first shown for tobacco (Hamberg *et al.*, 1999), and later described for several plant species including pea, *Arabidopsis* and rice (Fig. 7.2). The tobacco  $\alpha$ -DOX catalyzes the stereo-specific insertion of oxygen leading to unstable 2-hydroperoxide derivatives (Hamberg *et al.*, 1999). The  $\alpha$ -DOX expression is up-regulated upon bacterial infection of tobacco leaves, and metabolic profiling of tobacco leaves infected with an incompatible strain of *Pseudomonas syringae* revealed strong rise in accumulation of  $\alpha$ -DOX products and decrease in LOX products indicating role of  $\alpha$ -DOX in plant pathogen interactions (Hamberg *et al.*, 2003), oxidative stress and cell death (de Leon *et al.*, 2002).

### 7.2.2 Phytoprostanes and electrophile compounds

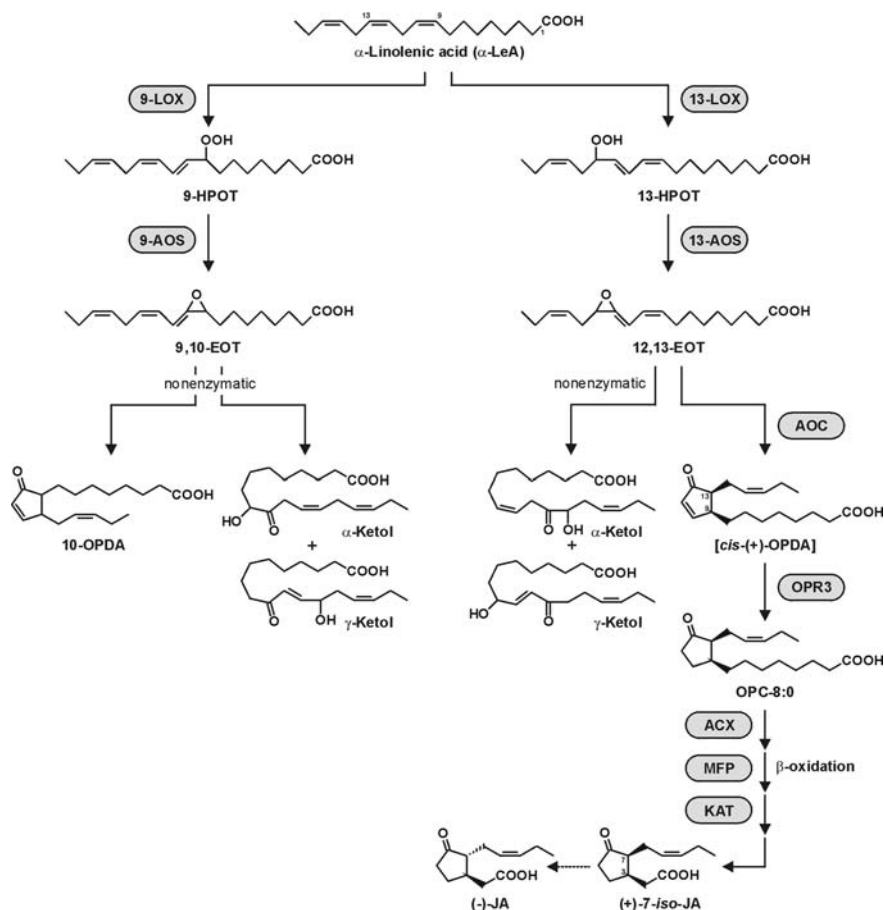
The plant-specific JA biosynthesis from  $\alpha$ -linolenic acid ( $\alpha$ -LeA) is homologous to prostaglandin biosynthesis from arachidonic acid in animals. Detailed inspection of degradation products of lipid membranes revealed the occurrence of numerous prostaglandin-like oxylipins called phytoprostanes. Phytoprostanes are formed non-enzymatically by action of free radicals. First products are 9(*R*, *S*)-, 12(*R*, *S*)-, 13(*R*, *S*)- and 16(*R*, *S*)-hydroperoxy fatty acids, whereas the enzymatically (LOX)-formed compounds are 9(*S*)- and 13(*S*)-hydroxyperoxy fatty acids (Mueller, 2004) (see Section 7.3.1). Most of these phytoprostanes are esterified in membranes and increase upon oxidative stress. Some of them were shown to be short-lived stress metabolites that mediate expression of genes known to function in stress protection and apoptosis (Loeffler *et al.*, 2005). Although most of lipid peroxidation is assumed to occur by LOXs and  $\alpha$ -DOX, phytoprostanes and non-enzymatically formed oxylipins are permanent constituents with significant amount in extracts of healthy plant tissues.

Wound- and pathogen-induced accumulation of fatty acid degradation production with a ketodiene or ketotriene structure (Vollenweider *et al.*, 2000) led to the concept that these types of compounds, for example malondialdehyde (MDA), represent reactive electrophile species with activity in plant-defense gene expression



**Figure 7.2** The different branches of the LOX pathway. Metabolism of  $\alpha$ -LeA by non-enzymatic formation of dinorisoprostanes,  $\alpha$ -dioxigenase ( $\alpha$ -DOX)-catalyzed formation of  $\alpha$ -hydroxy-PUFAs and 13-lipoxygenase (13-LOX)-catalyzed formation of the 13-hydroperoxide of  $\alpha$ -LeA (13-HPOT). 13-HPOT can be the substrate of a reductase, an EAS, a DES, an AOS, a POX, an LOX and an HPL. Adapted from Feussner & Wasternack, 2002.

(Farmer *et al.*, 2003). Transcriptome and metabolome analyses with *Arabidopsis* revealed remarkably different properties among the various electrophile species such as MDA and small vinyl ketones (Alméras *et al.*, 2003). Whereas MDA seems to act as a crosslinking/modifying agent thereby contributing to expression of genes in response to abiotic stress, the vinyl ketones strongly activate genes in response to biotic stress (Weber *et al.*, 2004). It will be interesting to see how the biological relevance of these reactive carbonyl compounds can be dissected by use of mutants. First indication came from analysis of the JA-deficient *opr3* mutant of *Arabidopsis*. Here, the ketodiene-containing OPDA accumulated in response to pathogen attack suggesting role of OPDA but not JA in some plant pathogen interactions (Stintzi *et al.*, 2001). Transcriptome analysis following wounding or treatment with OPDA,



**Figure 7.3** Metabolic scheme showing formation of 9-LOX and 13-LOX products from  $\alpha$ -LeA. In both pathways LOX products are used by AOSs with positional specificity. The corresponding epoxides are spontaneously hydrolyzed to  $\alpha$ - and  $\gamma$ -ketols, as well as racemic OPDA, whereas in the presence of AOC *cis*-(+)-OPDA is formed. EOT: epoxyoctadecatrienoic acid.

JA or MeJA revealed a remarkable number of genes that were specifically up-regulated by OPDA (Taki *et al.*, 2005).

### 7.3 The LOX pathway

PUFAs are constituents of membrane lipids, which are permanently altered in their composition and turnover. Following release of PUFAs hydroperoxides are synthesized by different forms of LOXs. The LOX pathway covers all LOX-catalyzed reactions, and the subsequent steps lead to different groups of oxylipins (Fig. 7.3).



### 7.3.1 The LOX

LOXs (linoleate: oxygen oxidoreductase, EC 1.13.11.12) catalyse the regio- and stereo-specific insertion of oxygen in PUFAs containing a (1Z,4Z)-pentadiene system, for example linoleic acid (18:2, LA) or  $\alpha$ -linolenic acid (18:3,  $\alpha$ -LeA). Oxygen insertion takes place either at C-9 (9-LOX) or at C-13 (13-LOX) of the carbon backbone leading with  $\alpha$ -LeA as substrate to (9S)-hydroperoxyoctadecatrienoic acid (9-HPOT) or (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) (Fig. 7.3). The exclusive formation of the *S*-isomer indicates enzymatic synthesis, whereas concomitant presence of the *R*-form is indicative for non-enzymatic synthesis. LOXs constitute a large gene family of non-heme iron-containing dioxygenases. Beside classification of plant LOXs according to positional specificity (9-LOXs, 13-LOXs), they can be grouped by their primary structure. LOXs lacking a transit peptide and exhibiting high-sequence similarity (>75%) are grouped as *type1*-LOXs, whereas LOXs with a putative chloroplast transit peptide sequence and low overall sequence similarity (~35%) are classified as *type2*-LOXs. All *type2*-LOXs identified so far are 13-LOXs. In respect to the reaction mechanism of LOXs, the space within the active site and the orientation of the substrate were identified to be important factors for positional specificity (Feussner & Wasternack, 2002). In the case of the cucumber lipid body 13-LOX, site-directed mutagenesis of the histidine residue within the substrate binding pocket led to a 9-LOX activity (Hornung *et al.*, 1999). In contrast to higher plants, lower plants such as algae or the moss *Physcomitrella patens* carry LOX enzymes with less substrate specificity and even a multifunctional LOX was found recently in *P. patens* (Senger *et al.*, 2005). This enzyme is a 12-LOX with arachidonic acid and a 13-LOX with LeA as substrate and even C<sub>18</sub>–C<sub>22</sub> fatty acids are partially tolerated. Surprisingly, this LOX is also a hydroperoxide lyase which allows *P. patens* to generate a broad spectrum of defense compounds by a single enzyme (Senger *et al.*, 2005). This dual function of a single enzyme is reminiscent to a dual-oxylin metabolism of red algae, where animal-like (eicosanoid) and plant-like (octadecanoid) oxylin seems to be components of defense reactions (Bouarab *et al.*, 2004).

The available sequence data allowed phylogenetic tree analysis which led to individual groups for all *type1*- and *type2*- as well as all 9- and 13-LOXs including distinction of monocotyledonous and dicotyledonous LOXs within these subgroups (Feussner & Wasternack, 2002). The clear distinction between LOXs on the basis of their regiospecificity is seen also with respect to expression pattern, intracellular location and substrate utilization. Furthermore, conversion of 9-LOX as well as 13-LOX products by enzymes specific for one of these products led to the concept that oxylin are formed in discrete 9-LOX and 13-LOX pathways (Howe & Schillmiller, 2002) (Fig. 7.3).

The 9-LOX and subsequent reactions seem to have an essential role in plant defense. Initially, an enhanced susceptibility to infection with *Phytophthora parasitica* was observed in tobacco plants with *antisense* expression of a specific 9-LOX. The potato homologue was activated upon infection of leaves (Kolomiets *et al.*, 2000), and is also activated in elicitor-treated potato cells grown in suspension

including elevation of 9-LOX products (Göbel *et al.*, 2001). Interestingly, in infected potato plants deficient in 9-LOX expression high level of non-enzymatically formed 9-LOX products were found, whereas wild-type plants contained 9-LOX-derived compounds (Göbel *et al.*, 2003), indicating that the hypersensitive response appearing in both plant types was independent of the source of lipid hydroperoxides.

Beside the obvious role of 9-LOX products in plant defense, developmental programmes seem to be controlled by a 9-LOX. A 9-LOX of rice is specifically expressed in germinating seeds (Mizuno *et al.*, 2003) and a tuber-specific 9-LOX (*StLOX1*) is expressed during tuber initiation. Tuber development was disrupted in plants with antisense expression of this 9-LOX (Kolomiets *et al.*, 2001) (see Section 7.6.2).

Among the potato and tomato 13-LOXs the potato LOXH3 and its tomato homologue TomloxD function in wound-induced defense signaling (Royo *et al.*, 1999), whereas the TomloxC, a homologue of potato LOXH1, is highly expressed in fruits and functions in generation of volatile C<sub>6</sub> flavour compounds (Chen *et al.*, 2004a).

### 7.3.2 HPOT/HPOD: the branch point in the LOX pathway

The majority of LOXs are 13-LOXs, which are localized in chloroplasts (Feussner *et al.*, 1995; Feussner & Wasternack, 2002). The 13-LOX product 13-HPOT formed by oxygenation of  $\alpha$ -LeA (18:3), and the 18:2 fatty acid oxygenation product 13-hydroperoxyoctadecadienoic acid (13-HOPD) are substrates of at least seven different enzymes, which initiate individual branches within the LOX pathway (Fig. 7.2):

- (1) The allene oxide synthases (13-AOSs) leading to octadecanoids and jasmonates.
- (2) The hydroperoxy lyases (13-HPLs) leading to  $\omega$ -oxo fatty acids, aldehydes and alcohols.
- (3) The peroxigenases (POXs) forming epoxy hydroxy-PUFAs.
- (4) The divinyl ether synthases (DESSs) leading to divinyl ether-containing PUFAs.
- (5) LOXs catalyzing the formation of keto-PUFAs.
- (6) The epoxyalcohol synthases (EASs) forming epoxyhydroxy-PUFAs.
- (7) The reductases leading to hydroxy PUFAs.

The corresponding reactions can take place with the 9-LOX products 9-HPOT and 9-HPOD (Figs. 7.2 and 7.3). Consequently, a remarkably high number of oxylipins exist. Since the various PUFAs, preferentially 18:2 and 18:3, and many of their oxygenation products occur in free and esterified form, a metabolite profiling of oxylipins to describe lipid peroxidation and lipid-derived signals requires large-scale high performance thin-layer chromatography (HPLC) analysis including separation of enantiomeric forms. So far, only few laboratories can perform such a large-scale metabolite profiling.

Many oxylipins generated by the different branches of the LOX pathway are antifungal and antimicrobial, for example the POX-generated epoxy and hydroxyl derivatives (Blée, 2002). This might be linked to a role of POX in cutin formation (Lequeu *et al.*, 2003). Also divinyl ethers generated by DES are elevated upon

pathogenic attack and are thought to possess antimicrobial activity (Weber *et al.*, 1999; Göbel *et al.*, 2001). Antifungal activity was also detected for EAS products of 9-HPOT (Hamberg, 1999). DES together with AOS and HPL are highly related members of the CYP74 family of P450s. They are characterized by lack of a requirement for O<sub>2</sub> and an external redox compound. Based on sequence identity and enzymatic properties including substrate specificity, CYP74 enzymes are grouped in the subfamilies A, B, C and D (<http://drnelson.utmem.edu/cytochromeP450.html>). AOSs and HPLs using 13-hydroperoxides as the substrate belong to the subfamily A and B, respectively, CYP74C enzymes are HPLs accepting 9- or 13-hydroperoxides, whereas CYP74D enzymes are DESs with specificity for 9-hydroperoxides (Feussner & Wasternack, 2002; Howe & Schilmiller, 2002). The role of HPLs in defense reactions has been repeatedly shown (see Sections 7.5.1.2 and 7.5.3). Whereas 13-HPL-derived C-6 volatiles exhibit antimicrobial activity and affect aphid performance (Croft *et al.*, 1993; Vancanneyt *et al.*, 2001), 9-HPL-derived nonenals may lead to a broad resistance to herbivores and pathogens. Interestingly, the moss *P. patens* contains not only a 9-LOX with HPL activity as mentioned above (Senger *et al.*, 2005), but also a HPL which converts 9-hydroperoxides of C-18 fatty acids and the corresponding 11-hydroperoxides of arachidonic acid, a constituent of *P. patens* (Wichard *et al.*, 2004). This may explain the natural broad resistance of *P. patens* against pathogens. It will be interesting to discover how the regulatory interplay between the HPL branch and the AOS branch determine the diverse patterns of oxylipins which could allow the plants to respond specifically to various environmental factors occurring under stress in nature.

### 7.3.3 The AOS branch: jasmonate biosynthesis

#### 7.3.3.1 The AOS

The initial reaction in the JA-forming branch of the LOX pathway is catalyzed by AOS. This enzyme, which was cloned first from flax (Song *et al.*, 1993; Pan *et al.*, 1995), is a cytochrome P450-type (CYP74A), but is independent of molecular oxygen and NADPH, and exhibits low affinity to CO. In the AOS-catalyzed reaction, the hydroperoxide group of LOX products is used as the source of reducing equivalents and of oxygen leading to a highly unstable epoxide, which degrades spontaneously to  $\alpha$ -ketol and  $\gamma$ -ketol or racemic 12-OPDA (Fig. 7.3). In the AOS gene family about 15 members have been cloned so far. Phylogenetic analysis indicates clear separation of the 9-AOSs specific for 9-hydroperoxides and the 13-AOSs specific for 13-hydroperoxides (Howe & Schilmiller, 2002), supporting the above-mentioned concept of discrete 9-LOX and 13-LOX pathways. The 9-LOX/9-AOS pathway was demonstrated for tulip bulbs (Grechkin *et al.*, 2000), potato (Hamberg, 2000; Stumpe *et al.*, unpublished information), and tomato (Itoh *et al.*, 2002). Its activity is increased upon pathogen attack (Göbel *et al.*, 2002) accompanied by increased generation of oxylipins, such as 10-oxo-11,15-phytodienoic acid (10-OPDA) (Hamberg, 2000). Among the various plant species, the number of genes encoding AOSs is different. Whereas *Arabidopsis* has a single copy gene

(Laudert *et al.*, 1996), there are three AOS genes in barley, potato and tomato (Howe *et al.*, 2000; Maucher *et al.*, 2000; Itoh *et al.*, 2002; Maucher *et al.*, 2004; Stumpe *et al.*, unpublished information). One of the three AOSs of tomato is a root-specific 9-AOS (Itoh *et al.*, 2002). The potato 9-AOS is also expressed in roots, and additionally in tubers and sprouting eyes, and the encoded protein located in the envelope of young amyloplasts catalyzes formation of  $\alpha$ -ketols (Stumpe *et al.*, unpublished information). This suggests a new role for this type of hydroperoxide degradation products which was overlooked so far due to missing analytical methods. The barley AOSs exhibit either preference for 13-hydroperoxides, or accept 9- and 13-hydroperoxides (Maucher *et al.*, 2000). Obviously, in some plants the AOS is defined by substrate and tissue specificity which is of regulatory impact. Due to the single AOS in *Arabidopsis*, the AOS-knockout mutant is JA deficient, lacks wound-induced accumulation of JA and JA-responsive gene expression and is male sterile, the characteristic phenotype of JA deficiency (see Section 7.4) (Park *et al.*, 2002; von Malek *et al.*, 2002). Except for the guayule AOS in rubber particles, all AOSs analysed so far were localized in chloroplasts, immunocytochemically and by import studies (Maucher *et al.*, 2000; Froehlich *et al.*, 2001; Stenzel *et al.*, 2003b). Furthermore, with the exception of the barley AOSs (Maucher *et al.*, 2000), they all exhibit a putative chloroplast target sequence (Song *et al.*, 1993; Laudert *et al.*, 1996; Froehlich *et al.*, 2001).

Upon wounding the AOSs are expressed locally and systemically in tomato, *Arabidopsis* and tobacco (Kubigsteltig *et al.*, 1999; Howe *et al.*, 2000; Kubigsteltig & Weiler, 2003). In contrast, *Arabidopsis* plants treated locally with OPDA, JA and their molecular mimic coronatine exhibited only local activation of the AOS promoter (Kubigsteltig *et al.*, 1999). The AOS promoter analyses in *Arabidopsis* showed also developmental regulation. High AOS promoter activity, detected in the anther filament and early stages of carpel development (Kubigsteltig *et al.*, 1999), points to role for JA in anther development, as clearly indicated by JA-biosynthesis mutants and JA-insensitive mutants (see Section 7.4). This accords with the tissue-specific occurrence of high amounts of various oxylipins, including JA, in tomato flower organs (Hause *et al.*, 2000).

First indications for regulatory elements upstream of the AOS promoter are given by the *cas1* mutant of *Arabidopsis* which exhibits elevated levels of AOS mRNA, AOS protein, JA and OPDA leading to constitutive expression of JA-inducible genes (Kubigsteltig & Weiler, 2003). So far, *CAS1* is not cloned, but might be a negative regulator of AOS expression. First transcription factors of JA-responsive gene expression were identified for *Catharanthus roseus* cells as AP2-domain transcription factors, such as ORCA3 (Van der Fits & Memelink, 2001). It will be interesting to see whether there are ORCA homologues in *Arabidopsis* which activate genes of JA biosynthesis such as AOS.

#### 7.3.3.2 The allene oxide cyclase

The spontaneous decomposition of the AOS product is by-passed by allene oxide cyclase (AOC)-catalyzed formation of *cis*-(+)-OPDA. AOC establishes the

enantiomeric structure of the naturally occurring cyclopentenones and cyclopentanones, such as JA. Since the first cloning of *AOC* from tomato (Ziegler *et al.*, 2000), more than 58 sequences are available, indicating the occurrence of gene families of different size (Stenzel *et al.*, 2003b). In contrast to the single copy gene of *AOS*, there are four *AOCs* in *Arabidopsis* (Stenzel *et al.*, 2003b), whereas in tomato the inverse situation occurs, a single copy gene for *AOC* and three *AOSs* (Howe *et al.*, 2000; Ziegler *et al.*, 2000). Further differences among the various plant species are given by tissue specificity. Whereas *LOX*, *AOS* and *AOCs* of *Arabidopsis* occur in all leaf tissues (Stenzel *et al.*, 2003b), the tomato *AOC* is confined to vascular bundles, the surrounding parenchymatic cells and the ovules of flower buds (Hause *et al.*, 2000) suggesting regulatory role (see Section 7.3.3.5). In fact, the high abundance of *AOC* in ovules of tomato flowers is correlated with high amount of octadecanoids and jasmonates in pistils (Hause *et al.*, 2000). In all species analysed so far, the *AOCs* are expressed upon wounding or other stimuli, such as glucose, osmotic stress or JA treatment (Howe *et al.*, 2000; Agrawal *et al.*, 2003; Stenzel *et al.*, 2003a & b). Interestingly, salt tolerance is given by a 70 amino acid extension of the mangrove *AOC*, which is up-regulated under salt stress (Yamada *et al.*, 2002). Less understood is the light dependency of JA formation. *AOC* might be a target of phytochrome regulation (Agrawal *et al.*, 2003; Riemann *et al.*, 2003). During development of roots and flowers the four *AOCs* of *Arabidopsis* are expressed in a spatially and temporally distinct, non-redundant manner (Stenzel *et al.*, unpublished information).

### 7.3.3.3 *OPR3*

The conversion of cyclopentenones into cyclopentanones is catalyzed by the OPDA reductase (*OPR*). This enzyme is encoded by small gene families in *Arabidopsis* and tomato (Strassner *et al.*, 2002). Interestingly, among the three *OPRs* of both species, only *OPR3* exhibits specificity for *cis*-(+)-OPDA (Schaller *et al.*, 2000; Strassner *et al.*, 2002), whereas the other *OPRs* seem to convert unspecifically  $\alpha$ -,  $\beta$ -unsaturated carbonyl compounds. The specificity of *OPR3* is strongly supported by identification of the JA-deficient lines *opr3* and *dde1* with mutations in *OPR3* (Sanders *et al.*, 2000; Stintzi & Browse, 2000), indicating that *OPR1* and *OPR2* cannot substitute the *OPR3* function. *OPR3* carries a peroxisomal target sequence and was shown to be localized in peroxisomes (Strassner *et al.*, 2002). *OPR3* expression is induced by numerous stimuli, such as wounding, and correlates with the accumulation of JA (Strassner *et al.*, 2002). Due to the location of *AOC* in chloroplasts and the peroxisomal location of *OPR3*, transport of OPDA or its CoA ester is required between these organelles. Indeed, there is evidence for the import of OPDA or its CoA ester by the ABC transporter COMATOSE (CTS), also known as PXA1. *CTS* mutants are JA deficient, but not male sterile indicating that the CTS function can be bypassed possibly by ion trapping of OPDA<sup>H</sup> (Theodoulou *et al.*, 2005).

### 7.3.3.4 $\beta$ -oxidation in JA biosynthesis

The final steps in JA biosynthesis require shortening of the carboxylic acid side chain of 12-oxophytoenoic acid (OPC-8). Feeding experiments with OPC-derivatives

carrying different lengths of the carboxylic acid side chain revealed that only even-numbered OPC derivatives are converted to JA suggesting  $\beta$ -oxidative side chain shortening (Miersch & Wasternack, 2000). Recently, genetic evidence indicated that, similar to auxin biosynthesis, the final steps of JA biosynthesis take place *via* fatty acid  $\beta$ -oxidation steps. Fatty acid  $\beta$ -oxidation requires the activity of an acyl-CoA synthase, an acyl-CoA oxidase (ACX), a multifunctional protein (MFP) and a L-3-ketoacyl CoA thiolase (KAT) (Fig.7.4). The *antisense* expression of *ACX1* and *KAT2* led to JA deficiency upon wounding (Castillo *et al.*, 2004). Cloning of *ACX1A* of tomato revealed its role in wound-induced JA biosynthesis, herbivore resistance and generation of a systemic wound signal (Li *et al.*, 2005). Interestingly, one of the 4-coumarate:CoA ligase-like enzymes of *Arabidopsis* is located in peroxisomes and can activate OPDA to the CoA ester (Schneider *et al.*, 2005). At present, two different scenarios are suggested:

- (1) OPDA or its CoA ester is transported into the peroxisomes by the ABC transporter CTS/PXA1 located in the peroxisomal membrane and further converted by OPR3, ACX1, MFP and KAT.
- (2) OPDA is transported into peroxisomes by ion trapping (Theodoulou *et al.*, 2005), directly reduced or activated by a 4-Cl-like enzyme (Schneider *et al.*, 2005) before being reduced by OPR3 and  $\beta$ -oxidized by ACX1, MFP and KAT (Fig. 7.4).

JA biosynthesis is thought to be regulated by three different factors:

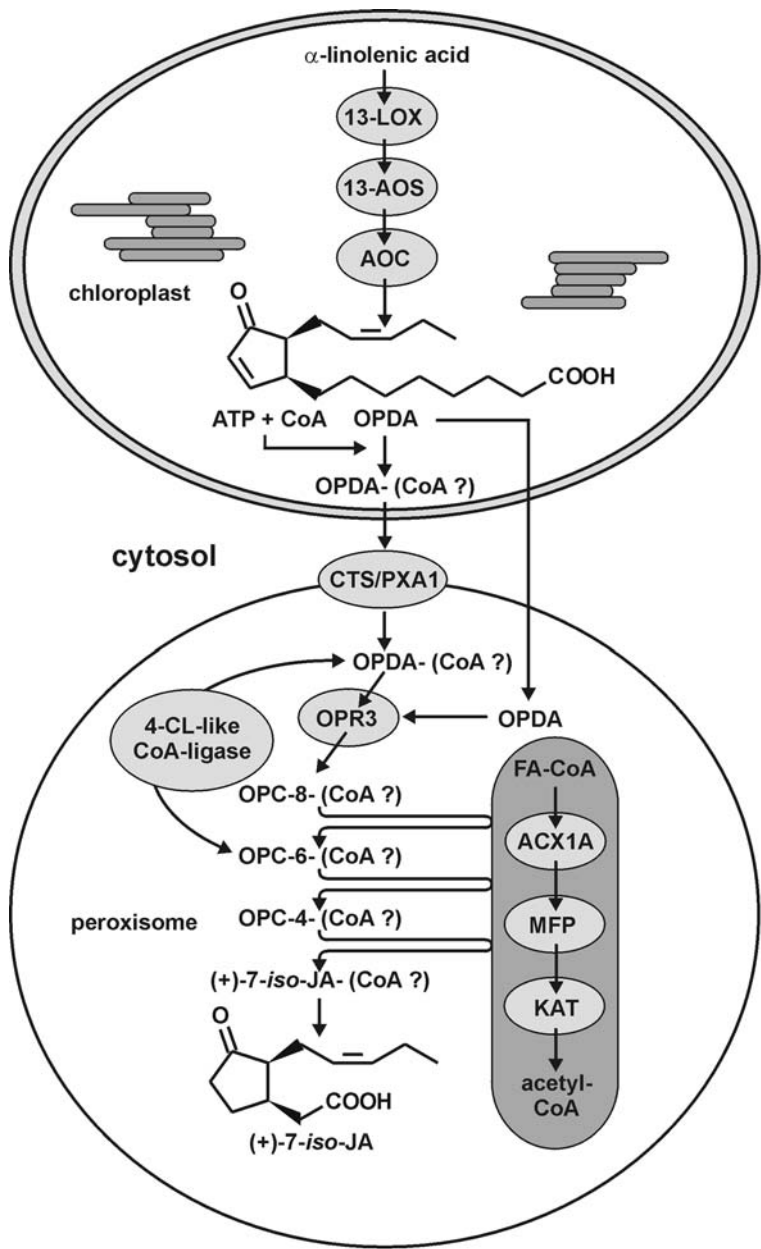
- (1) Substrate availability
- (2) Positive feedback
- (3) Tissue specificity.

The importance of substrate availability is indicated by the fact that transgenic plants, such as those of *Arabidopsis*, tobacco and tomato, over-expressing AOS or AOC constitutively, generate JA only upon wounding (Laudert *et al.*, 2000; Stenzel *et al.*, 2003a). This burst in JA precedes transcriptional activation of JA-biosynthetic genes (Laudert & Weiler, 1998; Stenzel *et al.*, 2003a & b). Furthermore, fully developed *Arabidopsis* leaves contain abundant LOX, AOS and AOC activities, but form JA only upon external stimuli such as wounding (Stenzel *et al.*, 2003b). Positive feedback in the regulation of JA biosynthesis was shown by (Stenzel *et al.*, unpublished information; Stenzel *et al.*, 2003b):

- (1) Up-regulation of JA-biosynthetic genes following treatment with JA.
- (2) Up-regulation of AOC expression in mutants with constitutively elevated JA levels.
- (3) A down-regulation of AOC expression in JA-deficient mutants such as *opr3*.

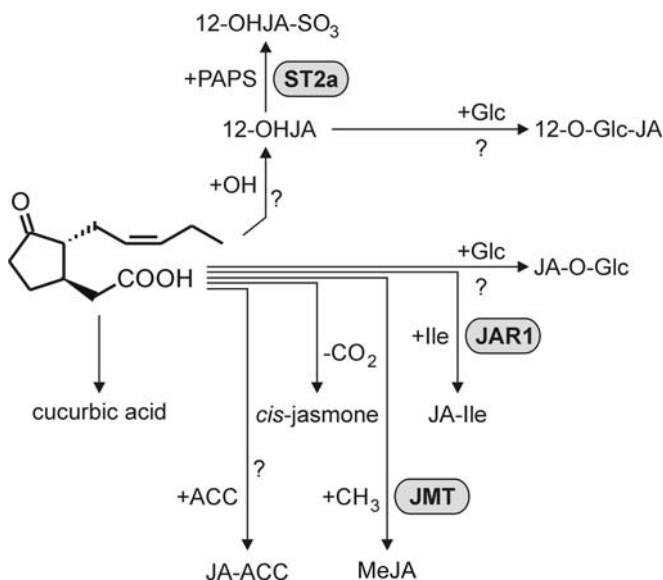
Finally, tissue-specific occurrence of JA-biosynthetic enzymes may affect the capacity to form JA. In tomato, AOC, but not LOX and AOS, is confined to vascular bundles while there is preferential formation of JA in this tissue upon wounding (Hause *et al.*, 2000; Stenzel *et al.*, 2003a). In *Arabidopsis*, the single-copy gene product





**Figure 7.4** Intracellular location of JA biosynthesis. The first half of JA biosynthesis is located in the chloroplast where OPDA is formed by the action of a 13-lipoxygenase (13-LOX), a 13-allene oxide synthase (13-AOS) and an allene oxide cyclase (AOC). Upon activation and transport into the peroxisomes, reduction by the OPDA reductase 3 (OPR3) and  $\beta$ -oxidation by fatty acid  $\beta$ -oxidation occur (see Section 8.3.3.4). OPC: oxopentenylcyclopentane. Adapted from Wasternack *et al.*, 2006.





**Figure 7.5** *Metabolism of JA*. The carboxyl group can be glucosylated, conjugated with amino acids, methylated, decarboxylated and conjugated with the ethylene precursor ACC. The pentenyl side chain can be hydroxylated and subsequently glucosylated or sulphated. The pentanone ring can be reduced to form cucurbic acids. So far the enzymes JA methyltransferase (JMT), JA conjugate synthase (JAR1) and 12-OHJA-sulphotransferase (ST2a) have been cloned from *Arabidopsis*.

AOS gives specificity for the AOS branch, but the four AOCs may allow the plant to generate OPDA and JA in a spatially and temporally distinct manner during development. As mentioned above, the four AOC promoters exhibited spatially and temporally different, non-redundant activities in root and flower development (Stenzel *et al.*, unpublished information).

### 7.3.3.5 Jasmonate metabolites

Accumulation of JA is usually taken as a first indicator for its role in any JA-dependent process under study. JA homeostasis, however, is influenced by at least six metabolic conversions most of them not well understood (Fig. 7.5):

- (1) Methylation at the carboxylic acid group by a JA-specific methyl transferase (Seo *et al.*, 2001).
- (2) Decarboxylation to *cis*-jasmone (Koch *et al.*, 1997).
- (3) Adenylation at the carboxylic acid side chain by an AMP transferase (Staswick *et al.*, 2002) followed by conjugation to an amino acid (Staswick & Tiryaki, 2004).
- (4) Hydroxylation at C-11 or C-12 of the pentenyl side chain followed by *O*-glucosylation (Sembdner & Parthier, 1993; Swiatek *et al.*, 2004b) or sulphation (Gidda *et al.*, 2003).

- (5) Formation of jasmonoyl-1- $\beta$ -glucose, jasmonoyl-1- $\beta$ -gentiobiose and hydroxyjasmonoyl-1- $\beta$ -glucose (Swiatek *et al.*, 2004b).
- (6) Reduction of the keto group of the cyclopentanone ring leading to cucurbitic acids (Sembdner & Parthier, 1993).

Methylation of JA by its methyl transferase is counteracted by unspecific esterases (Stuhlfelder *et al.*, 2004). Plant tissues contain much more JA than methyl jasmonate (MeJA). Constitutive over-expression of the JA-specific methyl transferase increased MeJA content without affecting that of JA, and was accompanied by a specific gene expression pattern and increased pathogen resistance (Seo *et al.*, 2001). This may indicate that under specific conditions the methyl ester is the active form of JA. Formation of the volatile *cis*-jasmane by decarboxylation is accompanied by resistance to insect feeding (Birkett *et al.*, 2000). Possibly, this is a direct and an indirect defense mechanism by *cis*-jasmane-induced emission of volatiles, repelling aphids and attracting aphid antagonists (see Section 7.5.3).

Similar to auxin amino acid conjugates, JA amino acid conjugates are formed by an enzyme which activates JA at the carboxylic acid group by adenylation followed by exchange of the AMP residue with any amino acid, but preferentially isoleucine (Ile) (Staswick *et al.*, 2002; Staswick & Tiryaki, 2004). The JA conjugate synthase is affected in the *jar1* mutant (see Section 7.4). This type of enzyme is encoded by a large gene family-containing members, such as auxin conjugate synthase (Staswick *et al.*, 2005), or 4-CL-like ligases (Schneider *et al.*, 2005). The predominant activity of JA conjugate synthase with Ile as the amino acid substrate accords with the preferential occurrence of JA-Ile among the JA amino acid conjugates in several plant species (Kramell *et al.*, 1997). JA-Ile is the most abundant oxylipin in tomato flowers (Hause *et al.*, 2000). Although in some species JA-Ile exhibits biological activity independent of JA (Kramell *et al.*, 1997), hydrolytic enzyme activity has been detected in some cases (Hertel *et al.*, 1997). Thus, for JA amino acid conjugates a similar situation seems to exist as for auxin conjugates, where auxin homeostasis is regulated by auxin conjugate synthase (Staswick *et al.*, 2005) and various auxin conjugate hydrolases (Rampey *et al.*, 2004).

Initially, hydroxylated JA and its *O*-glucoside were found exclusively in solanaceous species (Yoshihara *et al.*, 1989). 12-OH-JA was called tuberonic acid due to tuber-inducing properties, whereas its glucoside seems to be the transport form. 12-OH-JA synthesized in the leaves accumulates in tuber-forming stolons under short day conditions (Helder *et al.*, 1993). More recently, 12-OH-JA and its sulphated derivative have been found in *Arabidopsis* (Gidda *et al.*, 2003), while screening of many different dicotyledonous and monocotyledonous plants revealed the accumulation of 12-OH-JA in many species. In immature soybean seeds the 12-OH-JA level exceeds that of JA by up to 275-fold (Miersch *et al.*, unpublished information). Unlike JA, 12-OH-JA does not inhibit growth or tendrils coiling (Koda, 1992; Blechert *et al.*, 1999) and does not induce JA-responsive genes, such as *THIONIN2.1* in *Arabidopsis* (Gidda *et al.*, 2003), *PIN2* in tomato (Stenzel *et al.*, 2003a) or a set of JA-inducible genes of barley (Miersch *et al.*, 1999). These data suggest that hydroxylation of JA

might be a type of inactivation in JA signaling, at least for a subset of genes. For *Arabidopsis*, a sulphotransferase was cloned which converts specifically 12-OH-JA (Gidda *et al.*, 2003). Sulphation of hydroxylated compounds is a well-known mechanism in animal systems to inactivate hormones, suggesting hydroxylation of JA followed by sulphation as a route of complete inactivation of JA signaling.

The glucose ester of JA has been detected in cell cultures of tomato and tobacco (Meyer *et al.*, 1984; Swiatek *et al.*, 2004b). Detailed inspection of the tobacco BY-2 suspension culture revealed accumulation of 12-OH-JA, jasmonoyl-1- $\beta$ -glucose, jasmonoyl-1- $\beta$ -gentiobiose and hydroxyjasmonoyl-1- $\beta$ -glucose (Swiatek *et al.*, 2004b). Interestingly, these esters did not inhibit the G2 phase of the cell cycle, as has been shown for JA (Swiatek *et al.*, 2002) which blocks B-type cyclin-dependent kinase accumulation as well as *CYCLINB1;1* expression (Swiatek *et al.*, 2004a). This points again to inactivation of JA signaling by metabolism. It will be interesting to see whether or not these observations with cell suspension cultures can be confirmed with whole tissues. Initial data indicated a stimulatory effect of JA in meristematic tissues (Biondi *et al.*, 2001), and high AOC promoter activity was found in meristemic cells of the root tip (Stenzel *et al.*, unpublished information).

## 7.4 Mutants in JA biosynthesis and in JA signaling

### 7.4.1 Mutants in JA biosynthesis

Ten years ago the first mutant altered in JA biosynthesis was isolated. The triple mutant *fad3-2fad7-2fad8* is affected in the final step in the formation of  $\alpha$ -LeA, the substrate for JA biosynthesis. Due to the unique occurrence of  $\alpha$ -LeA in the tapetum of anthers,  $\alpha$ -LeA deficiency leads to JA deficiency. Consequently, the pollen fail to develop and dehisce properly leading to a male sterile phenotype (McConn & Browse, 1996). Subsequently, most of the *Arabidopsis* mutants affected in JA biosynthesis, including *aos*, *dde2-2*, *opr3*, *dde1* and *aim1*, were found to be male sterile (Table 7.1). In contrast, *dad1* and *comatose* plants are fertile due to residual JA formation. A characteristic phenotype of JA-deficient mutants of *Arabidopsis*, such as *dad1* and *opr3* is also an insufficient filament elongation at stage 12 of anther development (Stintzi & Browse, 2000; Ishiguro *et al.*, 2001; Mandaokar, 2003). Consequently, pollination occurs only upon JA treatment before stage 12. Expression analyses during anther development revealed up-regulation of *LOX2* (Mandaokar *et al.*, 2003), known to function in JA biosynthesis, and JA-responsive promoters are active in filaments (see Section 7.6.3). Possibly, the auxin-response factors ARF6 and ARF8 are involved in JA induction of filament elongation (see Section 7.6.3). A common phenotype of JA-deficient mutants is increased sensitivity to insects and pathogens, since both processes are JA dependent (see Section 7.5). Differential gene expression between *opr3* and wild-type plants has allowed identification of OPDA-dependent genes (Alméras *et al.*, 2003; Taki *et al.*, 2005). The tomato mutants *spr2* and *acx1* were found in a screen for suppressors of

**Table 7.1** Mutants and genes functioning in JA biosynthesis and JA signaling in *Arabidopsis* and tomato (modified from Lorenzo & Solano, 2005)

Mutants	Phenotype	Gene product	Reference(s)
<b>JA biosynthesis</b>			
<i>dad1</i>	Reduced filament elongation, delayed anther dehiscence JA deficient in flowers	Phospholipase A1	Ishiguro <i>et al.</i> (2001)
<i>fad3-2fad7-2fad8</i>	Male sterile, delayed anther development, altered $\alpha$ -LeA level	$\omega$ -7-fatty acid desaturase	McConn and Browse (1996)
<i>spr2<sup>1</sup></i>	Deficient in $\alpha$ -LeA and JA levels, no wound response suppressed <i>prosystemin</i> expression	$\omega$ -7-fatty acid desaturase	Li <i>et al.</i> (2003)
<i>aos</i>	JA deficient, decreased resistance to pathogens	AOS	Park <i>et al.</i> (2002)
<i>dde2-2</i>	Male sterile, delayed anther development,	AOS	Von Malek <i>et al.</i> (2002)
<i>opr3</i>	JA deficient, decreased resistance to pathogens, reduced filament elongation	OPR3	Stintzi and Browse (2000)
<i>dde1</i>	JA deficient, reduced filament elongation, delayed dehiscence1	OPR3	Sanders <i>et al.</i> (2000)
<i>acx1<sup>1</sup></i>	JA deficient reduced wound response	ACX	Li <i>et al.</i> (2005)
<i>aim1</i>	Anther development	MFP1	Richmond and Bleeker (1999)
<i>comatose</i>	Reduced JA content	COMATOSE/PXA1 ABC transporter	Theodoulou <i>et al.</i> (2005)
<b>Constitutive JA response</b>			
<i>cev1</i>	Constitutive expression of vegetative storage proteins	Cellulose synthase CeS3	Ellis and Turner (2001) Ellis <i>et al.</i> (2002)
<i>cet1-9</i>	Constitutive expression of thionins, increased JA levels	?	Hilpert <i>et al.</i> (2001)
<i>cex1</i>	Constitutive root growth inhibition, constitutive expression of JA-responsive genes	?	Xu <i>et al.</i> (2001)
<i>cas1</i>	Constitutive expression of <i>AOS</i>	?	Kubigsteltig <i>et al.</i> (2003)
<i>joe1</i>	Increased expression of <i>LOX2</i> , increased accumulation of anthocyanans	?	Jensen <i>et al.</i> (2002)
<i>joe2</i>	Reduced inhibition of root growth, increased expression of <i>LOX2</i>	?	Jensen <i>et al.</i> (2002)
<b>Others</b>			
<i>ore9</i>	Delayed leaf senescence	F-box protein	Woo <i>et al.</i> (2001)
<i>cos1</i>	Suppressor of JA-dependent defects in <i>coil</i> (root growth, senescence, defence)	Lumazine synthase	Xiao <i>et al.</i> (2004)

**Table 7.1** (Continued)

Mutants	Phenotype	Gene product	Reference(s)
<b>Reduced sensitivity to JA</b>			
<i>coi1</i>	Reduced root growth inhibition, male sterile, reduced filament elongation, enhanced sensitivity to necrotrophic pathogens	F-box leucine repeat (LRR) COI1	Feys <i>et al.</i> (1994) Xie <i>et al.</i> (1998)
<i>jai1</i> <sup>1</sup>	Female sterile, altered trichome development, increased sensitivity to pathogens, decreased wound response	Tomato homologue of COI1	Li <i>et al.</i> (2004b)
<i>jar1/jin4/jai2</i>	Reduced root growth inhibition by JA, increased sensitivity to necrotrophic pathogens	JA amino acid conjugate synthase	Lorenzo <i>et al.</i> (2004) Staswick <i>et al.</i> (1992) Staswick and Tiryaki (2004)
<i>jin1/jai1</i>	Reduced root growth inhibition	AtMYC2 (bHLHzip transcription factor)	Lorenzo <i>et al.</i> (2004)
<i>jai3</i>	Reduced root growth inhibition in <i>ein3</i> background		Lorenzo <i>et al.</i> (2004)
<i>jue1-3</i>	Reduced expression of <i>LOX2</i>	?	Jensen <i>et al.</i> (2002)
<i>oji</i>	Enhanced sensitivity to ozone, reduced root growth inhibition	?	Kanna <i>et al.</i> (2003)
<i>mpk4</i>	Dwarf phenotype, altered expression of JA- and SA-response genes	AtMPK4	Petersen <i>et al.</i> (2000)
<i>rcd1</i>	Reduced sensitivity to JA, ethylene and ABA, impaired in ozone signaling	RADICAL-INDUCED CELL DEATH1	Ahlfors <i>et al.</i> (2004)
<i>axr1</i>	Reduced root growth inhibition by JA	RUB-activating enzyme	Xu <i>et al.</i> (2002)
<i>jai4/sgt1b</i>	Reduced root growth inhibition in the <i>ein3</i> background	AtSGT1b	Lorenzo <i>et al.</i> (2004)

<sup>1</sup>Tomato mutants.

prosystemin expression, an essential component of the wound-response pathway. These mutants are affected in the final step of  $\alpha$ -LeA biosynthesis by the  $\omega$ -7-fatty acid desaturase and in the ACX.

#### 7.4.2 Mutants in JA signaling

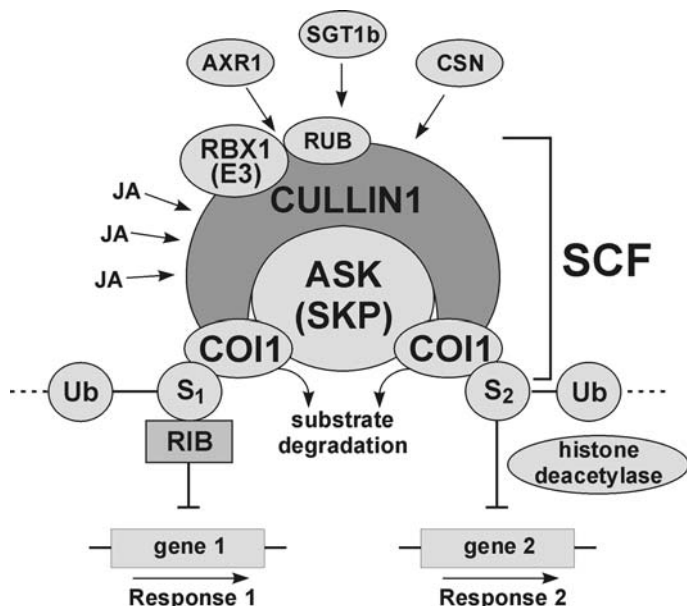
Mutants in JA signaling may lead to the following:

- (1) Reduced sensitivity or even insensitivity to JA or
- (2) Constitutive JA responses.

In the first group there is the most prominent member of JA-signaling mutants, *coi1*. Isolated in a screen for insensitivity to coronatine (Feys *et al.*, 1994), a molecular mimic of JA, the affected gene was shown to code for an F-box protein with function in ubiquitin-dependent protein degradation (Xie *et al.*, 1998) (see Section 7.4.3). Large-scale expression analyses with wild-type and *coi1* plants revealed the central role of *COI1* in JA-dependent gene expression (Reymond *et al.*, 2000; Devoto *et al.*, 2005). Together with data from the *opr3* mutant, genes were shown to be expressed *COI1* dependently, *COI1* independently, OPDA dependently, OPDA independently, JA dependently or JA independently (Devoto *et al.*, 2005; Taki *et al.*, 2005). The *coi1* plants are both male sterility and JA deficient. JA deficiency might be partially caused by the positive feedback regulation in JA biosynthesis (see Section 7.3.3.4). The tomato mutant *jai1*, in which a gene homologous to *COI1* is affected (Li *et al.*, 2004b), is female sterile and altered in trichome development, which indicates different functions of *COI1* in *Arabidopsis* and tomato (Howe *et al.*, 2004). A screen based on root growth inhibition by JA led to isolation of several *jar1* mutants (Staswick *et al.*, 1992), which are allelic to *jin4* and *jai2* (Lorenzo *et al.*, 2004). Cloning of *JAR1* revealed that JAR1 is homologous to adenylate-forming enzymes of the firefly luciferase family (Staswick *et al.*, 2002). JAR1 adenylates JA leading subsequently to conjugation with amino acids (Staswick & Tiryaki, 2004). JAR1 can also conjugate JA to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) suggesting a type of co-regulation in JA and ethylene availability. Interestingly, another allele of *jar1* encodes and is defective in auxin-amino acid synthase activity (Staswick *et al.*, 2005).

Cloning of the gene affected in *jin1* revealed that *JIN1* encodes the bHLHzip type transcription factor AtMYC2 (Lorenzo *et al.*, 2004). AtMYC2 is a positive regulator of JA-dependent, wound-induced expression of genes such as *VSP*, *LOX*, *Thi2.1* and a negative regulator of pathogen-defense genes, such as *PLANT DEFENSIN 2.1*, which are positively regulated by the ethylene-dependent transcription factor ERF1. Due to this antagonistic action of MYC2 and ERF1, plants can respond differentially to pathogen attack and wounding (Lorenzo & Solano, 2005). In development, AtMYC2 is a positive regulator of lateral root formation downstream of COI1 (Yadav *et al.*, 2005). A cross-talk between JA-dependent and salicylic acid (SA)-dependent signaling was found with *mpk4* (Petersen *et al.*, 2000). *MPK4* codes for the MAP kinase 4 which activates some JA-dependent defense genes and represses SA-dependent *PR* genes. The auxin-insensitive mutant *axr1* revealed the first cross-talk of JA- and auxin-signaling at the level of proteasome-mediated protein degradation (Xu *et al.*, 2002). *AXR1* encodes a RUB-activating enzyme which function in the SCF complex (see Section 7.4.3). Another member of these proteins was identified by the JA-insensitive mutant *jai4* screened in an ethylene-insensitive background (Lorenzo *et al.*, 2004). *JAI4* encodes a SGT1b and interacts like AXR1 with SCF subunits (Lorenzo & Solano, 2005).

Mutants with *constitutive JA response* were screened in plants carrying JA-responsive promoter reporter constructs (Table 7.1). All these mutants exhibit constitutive or elevated JA responses. In the case of *cas1*, a regulatory gene upstream



**Figure 7.6** Model of the SCF complex in JA signaling (modified from Dharmasiri & Estelle, 2004; using data of Devoto *et al.*, 2002; Xiao *et al.*, 2004). The constituents of the SCF complex CUL1, ASK1 (SKP1) and the F-box protein COI1 interact which each other. Upon conjugation of RUB1 by RBX1 (E<sub>3</sub>) to CULLIN1, the complex is active as E<sub>3</sub>-ligase leading to ubiquitination of COI1-attached substrates S<sub>1</sub> and S<sub>2</sub>. Consequently, ubiquitinated S<sub>1</sub> and S<sub>2</sub> are targeted to the proteasome and their negative control on gene1 and gene2 which mediate distinct JA responses (1,2) is abolished. In the case of S<sub>1</sub>, the negative control is dependent on a functional riboflavin pathway (RIB, lumazine synthase). A histone deacetylase might be a target of COI1. RUB conjugation to CUL1 is dependent on, or can be modulated by, other factors, such as the COP9 signalosome (CSN), SGT1b or AXR1, leading to pleiotropic effects.

of the AOS promoter seems to be affected. So far only the gene affected in *cevl* could be cloned. *CEV1* is the cellulose synthase gene *CeSA3*, which is preferentially expressed in roots, mid veins and anthers. Two other mutants (*ore9* and *cos1*) could be identified (Woo *et al.*, 2001; Xiao *et al.*, 2004); *ORE9* encodes an F-box protein, which delays senescence, whereas *COS1* encodes a lumazine synthase functioning downstream of COI1 (see Section 7.4.3; Fig. 7.6).

### 7.4.3 Proteasome-mediated JA signaling

In 1998, the *COI1* gene was identified as 67kDa protein with 16 leucine rich repeats (LRRs) and an N-terminal F-box motif (Xie *et al.*, 1998). F-box proteins occur ubiquitously in eukaryotic kingdoms and are known to recruit regulatory proteins for ubiquitin-dependent degradation in the proteasome. F-box proteins give the specificity for binding of the substrate which will be degraded upon ubiquitylation



by the other components of the SCF complex (Dharmasiri & Estelle, 2004). The SCF complex is composed of SKP1 (ASK1 in plants for *Arabidopsis* SKP1), CULLIN and RBX1 (for ring box1) as well as the F-box protein and functions as an E3 ligase (Fig. 7.6). All these SCF members occur in gene families, for example 19-SKP1-like proteins (ASKs) and about 700 potential F-box proteins (Gagne *et al.*, 2002).

CULLIN1 interacting with SKP1(ASKs) seems to function as a backbone and is modified with an ubiquitin-related protein 1 called RUB1. This modification is similar to the ubiquitylation, and is catalyzed by RBX1, which functions as a RUB-specific E3 ligase; CUL1 conjugated with RUB1 binds to ASK (Fig. 7.6). This model is discussed for ubiquitin-mediated protein degradation in developmentally regulated processes such as embryogenesis, hormone signaling and senescence, and in response to changes in the environment, such as light (circadian clock, photoperiod) (Moon *et al.*, 2004). The SCF complex functioning in auxin signaling is very well studied and led recently to the identification of its F-box protein (TIR) as the auxin receptor (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005).

The principle functions of the SCF components were also identified for JA signaling. Role of COI1 as an F-box protein could be identified by its physical interaction with CUL1, RBX1 and ASK1/ASK2 *in vitro* and *in vivo* and by the requirement of a functional F-box motif (Devoto *et al.*, 2002; Xu *et al.*, 2002). Furthermore, deficiency in any SCF component impairs JA signaling. A histone deacetylase (RPD3b) was identified as a putative target of COI1 (Devoto *et al.*, 2002). Another histone deacetylase (RPD3a) was shown to function in JA and ethylene signaling (Zhou *et al.*, 2005). Histone deacetylases are transcriptional repressors. In this scenario gene expression is induced by targeting negative regulators to protein degradation.

Whereas the numerous SCF-mediated responses can be explained in terms of the large number of F-box proteins, it is still unclear, how the numerous *COI1*-dependent processes are orchestrated. Beside a modular activity within the SCF complex, components upstream and downstream of *COI1* could allow individual *COI1*-dependent responses (Fig. 7.6). Regulation upstream of COI1 could occur by COP9. The COP9 signalosome (CSN), together with COP1 and COP10 is a key component of light-signal transduction occurring in photomorphogenetic processes. Surprisingly, CSN interacts also with E3 ligases, such as SCF<sup>COI1</sup>, and reduction in CSN functions affects JA signaling (Feng *et al.*, 2003), suggesting a link between JA and light signaling. CSN seems to affect also the amount of RUB1 conjugated to CULLIN1, as known for auxin signaling (Moon *et al.*, 2004). An example of a component downstream of COI1 is coronatine-insensitive suppressor 1 (COS1), which is a lumazine synthase, a critical component of riboflavin biosynthesis (Xiao *et al.*, 2004). Consequently, critical cellular processes using redox cofactors are affected in a *cos1* mutant. However, some *coi1*-related phenotypes (except male sterility) are restored in the mutant, suggesting that *COS1* (RIB) acts downstream of *COI1* and attributes to suppression of a negative regulator (substrate of SCF<sup>COI1</sup>) (Xiao *et al.*, 2004).

A link between JA signaling and components of plant responses to pathogens was found recently (Lorenzo & Solano, 2005). The *jai4* mutant (*cf.* Table 7.1) is

affected in a gene coding for SGT1b, which interacts with SCF complex components in yeast. SGT1b is a pleiotropic effector with roles in JA, auxin and pathogen signaling. Similarly, AXR1, a positive regulator of auxin responses, affects SCF activity *via* CULLIN1/RUB1 and leads to pleiotropic effects in JA signaling (Lorenzo & Solano, 2005). Consequently, the *axr1* mutant exhibits reduced root growth inhibition by JA. These examples highlight the central role of the ubiquitin-SCF-mediated protein degradation in orchestrating signaling pathways. Whereas COI1 acts specifically in JA signaling, other components allow cross-talk. Due to similarities in the function of SCF<sup>TIR</sup> and SCF<sup>COI1</sup> it is tempting to speculate, since TIR was identified as an auxin receptor (see above), that COI1 functions as a JA receptor.

## 7.5 JA, OPDA and related compounds in plant-defense reactions

### 7.5.1 Plant-microbe interactions

Plants have to cope with a great variety of microorganisms during their life-cycle. The interactions can be beneficial or deleterious for the plants. Symbiotic interactions occur by fungi leading to mycorrhiza or by bacteria, for example the nitrogen-fixing *Rhizobium spp.* interacting with legumes. Furthermore, there are non-pathogenic rhizobacteria which promote plant growth. In pathogenic interactions plants develop resistance by activation of a resistance gene (R) following recognition of an avirulence (AVR) gene product or by local restriction of pathogen growth (hypersensitive response, HR). In all these plant-microbe interactions JA was recognized to function as a signal.

#### 7.5.1.1 Symbiotic interactions

The formation of N<sub>2</sub>-fixing nodules in legumes by *Rhizobia* and the intracellular growth of fungi preferentially of the order *Glomales* defined as arbuscular mycorrhiza (AM) are two extensively studied symbiotic interactions between plants and microbes (Hause & Fester, 2005). Although *NOD* genes of *Rhizobia* are JA inducible (Rosas *et al.*, 1998), a role of JA in N<sub>2</sub>-fixation is not well studied. AM colonization of barley roots by the fungus was found to correlate with elevated levels of JA and expression of AOS within the arbuscule-containing cell (Hause *et al.*, 2002), whereas upstream components of the LOX pathway were not altered by AM (Stumpe *et al.*, 2005). Suppression of AOC in hairy roots of *Medicago truncatula* reduced JA levels and the degree of AM with *Glomus intraradices* indicating role of JA in the establishment of AM symbiosis (Isayenkov *et al.*, 2005).

The non-pathogenic rhizobacteria stimulates plant growth and induces systemic resistance (ISR) (Pozo *et al.*, 2004). Genetic studies with *Arabidopsis* infected by *Pseudomonas fluorescens*, *Xanthomonas campestris* or *P. syringae* revealed a JA- and ethylene-dependent pathway. This SA-independent ISR seems to be caused by enhanced sensitivity of the affected tissue to JA and ethylene, called "priming". Apart from a basal resistance, JA and ethylene elevate the expression of many

JA/ethylene-inducible genes such as *DEFENSIN* and *HEVEIN*. Primed plants are more effective in establishing resistance to the pathogen.

#### 7.5.1.2 Plant pathogen interactions

Plants can be attacked by a great diversity of pathogens. These are biotrophic, if living cells are required for pathogenic growth, or necrotrophic, if the attacked host cell is killed and used as the nutrient source for growth of the pathogens. Plants respond to biotrophic pathogens often by a hypersensitive response (HR), a SA-dependent response in which the attacked cell is killed by programmed-cell death (PCD) thus restricting pathogen growth. The various forms of establishment of plant innate immunity are ultimately linked with JA, SA and ethylene, although roles for ABA, brassinosteroids and auxins have also been shown. Obviously, the different resistance strategies have been evolved *via* a combinatorial use of these signaling compounds.

Beside being active in ISR (see Section 7.5.1.1), JA has a role together with ethylene in resistance against necrotrophic pathogens, such as the fungi *Alternaria brassicicola*, *Bothrytis cineria*, *Pythium* sp. and *Fusarium oxysporum*, and the bacteria *Erwinia carotovora*. This could be clearly shown by mutants of JA biosynthesis and of JA signaling (Berger, 2002; Kunkel & Brooks, 2002). JA-deficient *Arabidopsis* mutants, such as *fad3-2fad7-2fad8* exhibit increased susceptibility to *Pythium mastophorum* which could be compromised by JA treatment (Vijayan *et al.*, 1998). Similar data for the JA-insensitive mutants *coi1* and *jar1* support the JA dependence of the interactions (Staswick *et al.*, 1998).

Beside JA-deficient and JA-insensitive mutants, JA-accumulating mutants show altered response to necrotrophic pathogens. Increased resistance and constitutive expression of defense genes were found in *cev1* and *cet1* plants (Ellis & Turner, 2001; Hilpert *et al.*, 2001; Ellis *et al.*, 2002; Nibbe *et al.*, 2002). There is a common action of JA and ethylene in the response to many necrotrophic pathogens. An exception is *E. carotovora*, elicitors from which induce tryptophan biosynthesis and formation of its terminal product, the 3-indolylmethylglycosinolate, in a JA-dependent manner. Consequently, *coi1* plants are more sensitive to this pathogen, but not the ethylene-insensitive mutants (Brader *et al.*, 2001).

Similar to the common action of JA and ethylene in response to many necrotrophic pathogens, both signals act coordinately in response to abiotic stress, such as ozone exposure and wounding. The ozone-induced cell death of *Arabidopsis* is sustained by a mutual antagonism of ethylene and JA (Overmyer *et al.*, 2003; Tuominen *et al.*, 2004). An *rcd1* mutant affected in H<sub>2</sub>O<sub>2</sub> and ozone signaling is less sensitive to JA, ethylene and ABA (Table 7.1), and was identified as a member of the ADP-ribosyl transferase domain-containing subfamily of the WWE protein-protein interaction domain protein family. Thus, RCD1 may function in post-translational modifications as an integral mode in hormone signaling related to regulation of stress-responsive genes (Ahlfors *et al.*, 2004). Other reactive oxygen species, such as singlet oxygen (<sup>1</sup>O<sub>2</sub>) are also linked to PCD and ethylene/JA signaling. In contrast to the JA-induced suppression of H<sub>2</sub>O<sub>2</sub>/superoxide-dependent

PCD, JA promotes singlet-oxygen-induced PCD in the *flu* mutant, but is antagonized by OPDA (Danon *et al.*, 2005).

Phytopathogenic bacteria, such as *X. campestris* and *P. syringae* contain numerous genes that encode potential virulence factors. Interestingly, one of these factors, coronatine, is a molecular mimic of JA (Feys *et al.*, 1994) and leads to similar, but not identical gene expression profiles in the host (Uppalapati *et al.*, 2005). Recently, it was shown that coronatine, together with the *P. syringae* type III effectors, promotes parasitism by augmenting a *COI1*-dependent pathway in the host (He *et al.*, 2004). In tomato this coronatine-mediated, increased bacterial virulence is JAI1-dependent and suppresses *PR1* gene expression (Zhao *et al.*, 2003). In nature, plants are attacked simultaneously by various pathogens and insects. Many of them were shown to activate the JA-signaling pathway suggesting its dominant role in the establishment of resistance against diverse pathogens (Thaler *et al.*, 2004).

#### 7.5.1.3 Cross-talk between JA, SA, ethylene and ABA

Analyses of numerous *Arabidopsis* mutants revealed existence of two distinct signaling pathways in response to biotrophic and necrotrophic pathogens (Kunkel & Brooks, 2002). Whereas plants activate *PR* gene expression by a SA- and NPR1-dependent pathway in response to biotrophic pathogens, expression of defense genes, such as *DEFENSINS* and *THIONINS* is activated in a JA- and ethylene-dependent manner in response to necrotrophic pathogens (Glazebrook, 2001). There is, however, clear evidence for cross-talk between these signaling pathways by antagonistic and synergistic interactions. A mutual antagonism seems to occur preferentially. This can be a negative effect of SA on JA biosynthesis, and of cytosolic NPR1 on JA signaling (Spoel *et al.*, 2003). Furthermore, the WRKY70 transcription factor has been identified as a node of convergence for the SA- and JA-mediated signaling pathways (Li *et al.*, 2004a). Without affecting SA or JA levels, WRKY70 acts positively in the SA-mediated pathway downstream of NPR1, and negatively in the JA-mediated pathway downstream of COI1. Furthermore, the negative effect of SA on JA signaling could be demonstrated in mutants impaired in SA accumulation, such as *eds4* and *pad4*, or SA-accumulating mutants, such as *cpr6*. A negative effect of JA on SA signaling was shown in mutants, such as *mpk4*, *ssi2* and *coi1* that have enhanced or constitutive SA-dependent defense (Kunkel & Brooks, 2002). Few examples exist on synergistic action of SA and JA in response to pathogens (Schenk *et al.*, 2000) and a recently performed detailed inspection revealed antagonistic and synergistic action depending on the concentrations used (Mur *et al.*, 2006).

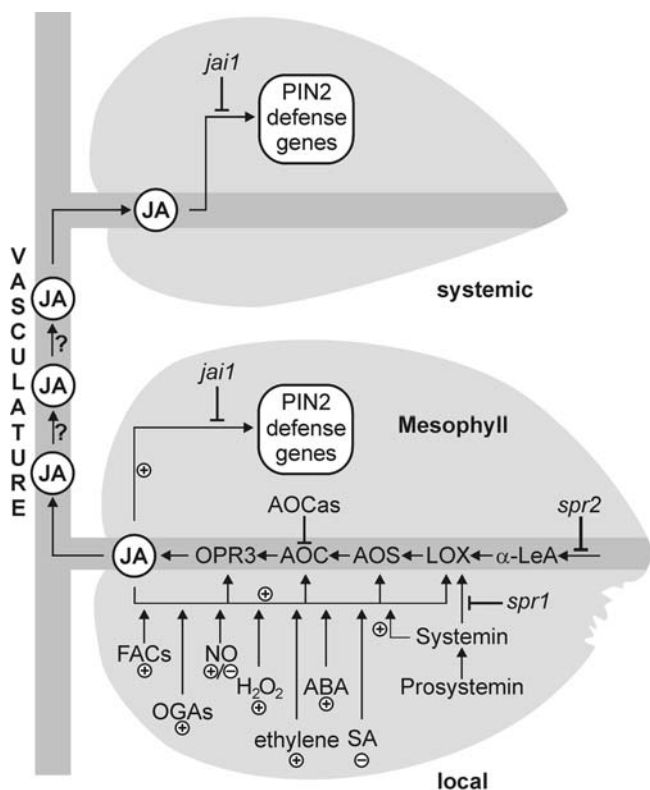
Possibly metabolic conversion of JA and/or SA may influence their signaling properties, for example loss of a SA-specific methyl transferase function influences the response to pathogens (Forouhar *et al.*, 2005). A recent transcriptional profiling of *Sorghum bicolor* treated with SA, JA or an ethylene precursor revealed numerous genes expressed synergistically, whereas others were antagonistically activated (Salzman *et al.*, 2005). This large-scale approach provided indications of how molecular switches in the SA- and JA-dependent signaling pathways might be orchestrated. As in the wound response, JA and ethylene function coordinately in the host

reactions to pathogens (Penninckx *et al.*, 1998). In contrast to the mutual antagonism of JA and ethylene in the plant response to ozone, which seems to occur by protein–protein interactions, their action in response to necrotrophic pathogens and wounding might be sustained at the level of transcription factors. The recently cloned *JIN1* affected in the JA-insensitive mutant *jin1* (see Section 7.4) codes for the transcription factor AtMYC2, which antagonizes ERF1, and both of them function downstream of COI1 (Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Lorenzo & Solano, 2005). The JA/ethylene pathway is also activated during non-host interactions (Zimmerli *et al.*, 2004).

An interesting sequential action of JA, ethylene and SA was shown for tomato responding to *X. campestris* (O'Donnell *et al.*, 2003), in a manner clearly different from that in *Arabidopsis*. Whereas *R*-gene-mediated resistance was not affected, the extent of HR was controlled sequentially by all three signals. In nature biotic stress by pathogens and abiotic stress, such as desiccation, may occur simultaneously. In *Arabidopsis* these responses to pathogens and stress are orchestrated by the antagonistic action of ABA on the JA/ethylene-signaling pathways (Anderson *et al.*, 2004).

### 7.5.2 The wound-response pathway

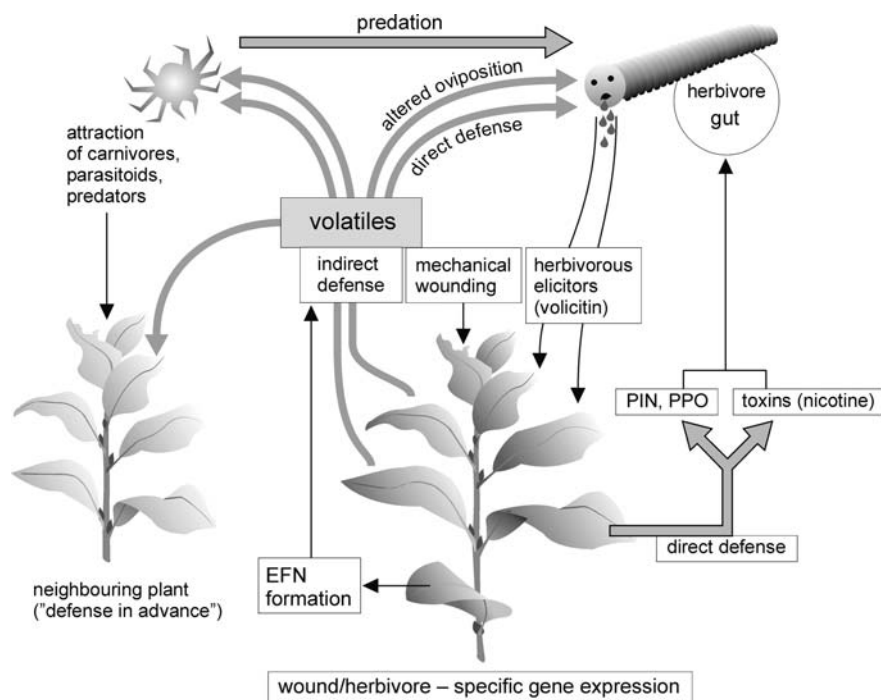
Many insects and other animals take nutrients from plant leaves or roots, which is accompanied with wounding. Plants respond with local and systemic formation of defense proteins, such as proteinase inhibitors (PINs) (Fig. 7.7), defense compounds, such as nicotine or volatiles influencing plant–plant and plant–insect interactions (Fig. 7.8, see Section 7.5.3). In all these wound responses JA is an essential signal. The wound-response pathway is the best-studied JA-signaling pathway, particularly in tomato and other solanaceous species, in which the accumulation of PINs upon wounding was first observed (Green & Ryan, 1972). In the early 1990s, work in Ryan's laboratory revealed that airborne MeJA can induce *PIN* expression (Farmer & Ryan, 1990), and the 18-amino acid peptide systemin was identified as an upstream signal (McGurl *et al.*, 1994). Meanwhile, a tremendous amount of data has accumulated (summarized in Fig. 7.7), but the JA receptor is still missing. In tomato, JA perception seems to occur at the plasma membrane, where applied labeled JA was localized (Bücking *et al.*, 2004). Herbivorous insects wound leaves. Mechanical wounding if applied alone is qualitatively similar to wounding by herbivores in terms of local and systemic responses (Mithöfer *et al.*, 2005). Herbivory, however, is accompanied by oral secretions from the feeding larvae containing a complex mixture of elicitors, including fatty acid amino acid conjugates (FACs) (Kessler & Baldwin, 2002; Halitschke & Baldwin, 2004). Among FACs the  $\alpha$ -LeA-glutamine conjugate (volicitin) was identified as highly active compound (Alborn *et al.*, 1997). FACs amplify wound signaling. Systemin is locally processed from a prosystemin precursor, which is encoded by a gene expressed in parenchymatic cells of vascular bundles (Narváez-Vásquez & Ryan, 2004). Subsequent systemin perception occurs at the systemin receptor, characterized as a protein of 160 kDa (SR160), but not yet localized. SR160 is a leucine-rich repeat receptor kinase with dual function



**Figure 7.7** The wound-response pathway in tomato. Local wounding of a leaf activates systemin processing and genes encoding enzymes of JA biosynthesis in the vascular bundles. Beside a positive feedback by JA, numerous signals act synergistically (+) or antagonistically (−) in JA generation (see Section 8.5.2). *PIN* expression is induced locally and systemically in the mesophyll cells. The systemic signal might be JA. Mutants known for tomato are indicated.

in systemin and brassinosteroid perception (Montoya *et al.*, 2002). Systemin perception may occur in neighbouring cells of the *PROSYSTEMIN*-expressing parenchyma cells, for example the companion cells, where JA-biosynthetic enzymes, such as AOC and its mRNA accumulation were localized (Hause *et al.*, 2003). Indeed, AOC expression in tomato is systemin dependent (Stenzel *et al.*, 2003a). Intracellular steps between systemin perception and AOC expression in the local tissue are documented by findings, such as wound-induced *PLA*<sub>2</sub> activation (Narváez-Vásquez *et al.*, 1999),  $\alpha$ -LeA accumulation (Conconi *et al.*, 1996a), elevated  $\text{Ca}^{2+}$  levels (Moyen & Johannes, 1996) and activation of MAPKs (Stratmann & Ryan, 1997). AOC expression may lead to amplification in wound signaling due to the JA-dependent *PROSYSTEMIN* expression (McGurl *et al.*, 1994) and systemin-dependent AOC expression (Stenzel *et al.*, 2003a). Most of these data were drawn from transgenic approaches covering sense and antisense expression of *PROSYSTEMIN*





**Figure 7.8** Direct- and indirect-defense mechanisms. Mechanical wounding or wounding by herbivores lead to expression of defense genes and defense compounds, such as nicotine, which are deleterious for the herbivore. Herbivorous regurgitants lead to additional or different responses including formation of volatiles that affect neighbouring plants, predators and herbivore oviposition. Adapted from Wasternack & Hause, 2002.

(McGurl *et al.*, 1994) and AOC (Stenzel *et al.*, 2003a), and from mutants affected in JA biosynthesis, JA signaling and systemin perception (Table 7.1). Upon local wounding a transient burst of JA occurs in the first hour followed by late events, which include expression of JA-biosynthetic genes and plant-defense genes, such as *PIN*s. Obviously, *PIN* expression is triggered spatially and temporally: whereas signals, such as JA and systemin are formed in vascular bundles, *PIN* expression occurs only in mesophyll cells of a leaf. Since JA and systemin are assumed to be insufficiently mobile, additional signals may attribute to *PIN* expression upon local wounding. One candidate is  $H_2O_2$ , which is highly mobile in the apoplast between vascular bundles and mesophyll cells and induces *PIN* expression (Orozco-Cárdenas *et al.*, 2001). Although the preferential and direct function of JA in local wound signaling has been substantiated by many studies, several other signals may modulate *PIN* expression indirectly (Schilmiller & Howe, 2005). Apart from systemin and FACS, these include ethylene, cell wall oligogalacturonides (OGAs), ABA, SA, NO, ROS (Fig. 7.7) (Ryan, 2000; Howe, 2004; Schilmiller & Howe, 2005). For ethylene and JA a synergistic action in the wound response was shown initially in tomato



(O'Donnell *et al.*, 1996), and has subsequently been observed in many other plants. Analyses of ABA-deficient tomato mutants revealed that ABA is also a signal of the wound response, presumably acting upstream of JA (Peña-Cortés *et al.*, 1995). SA was shown to inhibit wound-induced JA formation and *PIN* expression (Peña-Cortés *et al.*, 1995), but may also act downstream of JA formation. Synergistic and antagonistic action between these signals, depending of the plant system has been observed (Howe, 2004). OGAs were among the first signals identified to function in the wound response (Ryan, 1992) and seem to be generated by the wound-inducible polygalacturonase (Bergey *et al.*, 1999). The role of the immobile OGAs in the generation of the mobile  $H_2O_2$  was shown (Orozco-Cárdenas *et al.*, 2001). NO may negatively modulate the wound response in tomato (Orozco-Cárdenas & Ryan, 2002). This could be linked to the wound- and JA-inducible expression of *ARGINASE*, which in turn antagonizes NO production (Chen *et al.*, 2004b). Furthermore, UV light leads to *PIN* expression via the octadecanoid pathway (Conconi *et al.*, 1996b). The complex network in wound signaling is illustrated by the fact that these signals act sequentially, synergistically and antagonistically. Furthermore, some wound-induced genes are expressed JA-independently (Yalamanchili & Stratmann, 2002; Lee & Howe, 2003; Groß *et al.*, 2004). This fact highlights the flexibility of plants to respond on one external stimulus by expression of individual sets of genes. Recent large-scale expression analysis in *Arabidopsis* revealed that a large group of wound-induced genes were expressed in an OPDA- but not JA- or COI1-dependent manner, whereas others dependent on JA and COI1 (Devoto *et al.*, 2005; Taki *et al.*, 2005).

A characteristic feature of the wound response is an activation of plant-defense genes in systemic leaves distant from the wounded site. Several scenarios have been proposed for the nature of the systemic signal and the question, whether or not *de novo* signal generation occurs in the systemic leaves. Systemin, JA and/or related compounds, electric currents, or turgor pressure were suggested to act as systemic signals in tomato. Elegant reciprocal grafting experiments of G. Howe's laboratory led to strong arguments for JA as an essential component of the systemic signal. Using mutants affected in JA biosynthesis, such as *def1*, *spr2*, and *acx1*, in JA signaling, such as *jail*, and in systemin-signaling, such as *spr1* (see Section 7.4), it could be shown that the response in the systemic leaf does not require JA biosynthesis, OPDA perception and systemin perception, but JA perception is essential (Li *et al.*, 2002; Li *et al.*, 2005). Consequently, JA may be the mobile signal. The amplified generation of JA in vascular bundles (see above), the occurrence of LOX, AOS and AOC in sieve elements (Hause *et al.*, 2003) and the timing in the transport of phloem-mobile compounds support this assumption. Similar grafting experiments with the *coil* and *opr3* mutants suggest also a systemic activity of JA for *Arabidopsis* (Hawkes & Turner, 2004).

### 7.5.3 Direct and indirect defense

Despite numerous physiological and chemical data on "allelopathic" interactions between plants this concept was treated with scepticism for a long time. However,

the number of studies showing such interactions, as for example, altered leaf chemistry in trees neighbouring a wounded tree (Baldwin & Schultz, 1983) or induced formation of PINs in tomato leaves by airborne MeJA (Farmer & Ryan, 1990), has grown exponentially. Chemical communication between plants is now well accepted as a strategy for defense and fitness. Defense responses of plants, induced for example by piercing, chewing or sucking insects, can be established by formation of the following:

- (1) Proteins, such as PINs that are deleterious for the feeding insects
- (2) By synthesis of phytoalexins, such as nicotine
- (3) By formation and release of volatile organic compounds (VOCs) that attract parasitoids and predators
- (4) By formation and release of green leafy volatiles (GLVs)
- (5) By extra-floral nectar (EFN) formation
- (6) By a mechanical strategy such as increased cell wall thickness.

Consequently, these defense responses can be divided into direct and an indirect defense (Fig. 7.8). In both cases, JA acts as an essential signal. Direct defense is established following herbivore attack by formation of deleterious proteins, such as PINs or polyphenol oxidase (see Section 7.5.2), and formation of toxic quantities of secondary metabolites, such as nicotine or glucosinolates. Both of these responses are JA inducible. Although the nitrogen-intensive synthesis of compounds, such as nicotine may seem costly, plants are permanently monitoring the relative costs and benefits (Heil & Baldwin, 2002). Under field conditions the inducible-defense system established by jasmonates depends on herbivore pressure. In contrast to greenhouse experiments, several JA-inducible defense responses are constitutive in plants grown under field conditions. Furthermore, plants can recognize specific herbivores and can switch off the costly JA-inducible direct-defense mechanism, for example *Manduca sexta* larvae attacking *Nicotiana attenuata* release volicitin into the wound site leading to transcriptional down-regulation of direct-defense and up-regulation of indirect-defense marked by release of volatile compounds.

The indirect defense is given by release of a specific blend of volatiles, which attracts predators, such as parasitoids and carnivores, and the essential role of JA and SA in this process has been shown also for *Arabidopsis* (Van Poecke & Dicke, 2002). The emitted volatiles can be LOX-pathway-derived GLVs, such as leaf aldehydes, leaf alcohols or numerous monoterpenoids, sesquiterpenoids and diterpenoids. The specific pattern of compounds allows parasitoids to find their host. Such volatile emission correlating with high JA levels is elevated under nitrogen deficiency suggesting that plants are more sensitive to establish defense during lack of nutrients (Schmelz *et al.*, 2003). In case of GLVs, neighbouring plants can be primed against insect attack ("defense in advance") (Arimura *et al.*, 2000; Engelberth *et al.*, 2004) (Fig. 7.8). Spider mite-induced emission of volatile terpenoids and methyl salicylate is mediated by JA (Ament *et al.*, 2004). In the case of stem-boring insects on conifers, a similar but not identical pattern of terpenoids compared to that upon JA-treatment was found indicating a role of JA in resinosis and conifer defense (Miller *et al.*, 2005).

An interesting and intensively studied aspect of direct and indirect plant-defense strategies is how plants cope with the diversity of insects and how they orchestrate general and specific responses. On the one side, insects with completely different dietary strategies (specialists and generalists, such as *Spodoptera* and *Pieris*, respectively) induce a highly similar set of genes (Reymond *et al.*, 2004). On the other hand, herbivores can monitor intactness of host-defense signaling pathways. As shown with LOX-deficient plants in the native environment, *N. attenuata* was more sensitive to its specific herbivore, but also attracted novel herbivore species (Kessler *et al.*, 2004). This indicates that the composition of the herbivore community is dependent on the plant-signaling pathway in induced-defense responses. Besides the emission of VOCs and GLVs, EFN formation is a mechanism by which plants establish indirect defense (Heil *et al.*, 2001). Again, this strategy is mediated by the taxonomically conserved octadecanoid pathway. Symbiotic ants live on glands of the leaf stalk in many plants leading to resistance of the plant against herbivores. Interestingly, a phylogenetic analysis of the genus *Acacia* and closely related genera revealed constitutive EFN formation in those species on which ants are an obligate symbiont (Heil *et al.*, 2004). Obviously, inducible EFN formation has evolved to be constitutive by modified functional demands (Heil *et al.*, 2004). These plant–plant and plant–insect communications mediated by JA on the plant side exhibit a remarkable phenotype plasticity in the natural environment (Agrawal, 2001; Dicke *et al.*, 2003).

## 7.6 JA in development

Inhibition of germination and seedling development as well as leaf senescence were among the first physiological effects observed for JA applied to plants. Subsequently, mutant analyses revealed a role of JA in root growth and flower development (see Section 7.4). Tendril coiling and tuberization are further processes affected by JA or its metabolites.

### 7.6.1 Seedling development and root growth

Initial data on the role of JA in seedling development came from treatment with JA. Inhibitory effects appeared in non-dormant seeds, whereas seed germination of dormant seeds was stimulated by JA. Dry seeds and young seedlings exhibit high levels of JA. Interestingly, its metabolite 12-OH-JA can accumulate to much higher levels than JA (Miersch, personal communication). During seedling development, levels of jasmonates decline within a few days. So far the role of jasmonates in seedling development is not clear. They might be involved in

- (1) Growth and development by cross-talk with other hormones such as auxin, ethylene and ABA
- (2) Mobilization of reserve compounds

- (3) Increased defense status by direct action via their antimicrobial activity or by expression of defense genes (Wasternack & Hause, 2002).

Inhibition of root growth was one of the first physiological effects observed for MeJA (Koda, 1992) and has been used to isolate JA-insensitive mutants (see Section 7.4) (Berger *et al.*, 1996; Staswick *et al.*, 1992). Stunted roots following MeJA treatment correspond to the root phenotype of JA-accumulating mutants, such as *cev1* and *cet1* (Table 7.1). The molecular mechanism of root growth inhibition by JA is unclear. It might be based on the inhibitory effect of JA on auxin-induced cell elongation. Interestingly, the auxin-insensitive mutant *axr1* exhibits reduced root growth inhibition by JA supporting the cross-talk of auxin and JA *via* the SCF complex (Xu *et al.*, 2002) (see Section 7.4). Furthermore, the auxin-inducible *AXR2/IAA7* gene which encodes a negative regulator of auxin signaling (Nagpal *et al.*, 2000) is repressed by MeJA treatment (Devoto *et al.*, 2005). Consequently, generation of JA in the elongation zone, where *AOC* promoters are active (Stenzel *et al.*, unpublished information) would enhance auxin signaling by down-regulation of *AXR2/IAA7*. Lateral root formation seems to be JA dependent. *AtMYC2* which is affected in the mutant *jin1* (Table 7.1) is a key player in JA-induced gene expression. *AtMYC2* is transiently up-regulated by JA in a *COI1*-dependent manner (Lorenzo *et al.*, 2004) and is a positive regulator of lateral root formation (Yadav *et al.*, 2005). Correspondingly, lateral root primordia exhibit high-*AOC* promoter activity (Stenzel *et al.*, unpublished information).

### 7.6.2 Tuber formation

Tubers of potato are formed under short-day conditions and cool night temperature. The complex developmental programme from growing stolons to the swelling tubers is clearly affected by JA and its metabolite 12-OH-JA, called tuberonic acid (Koda, 1992; Wasternack & Hause, 2002). Consequently, a role of a tuber-specific *StLOX1* in tuber formation has been shown (see Section 7.3.1). Elevated levels of JA and 12-OH-JA formed in the leaves and transported to the stolons may induce cell expansion, a known phenomenon for jasmonates. Under long-day conditions, there is no tuber formation, and 12-OH-JA levels are undetectable (Helder *et al.*, 1993). The tuber formation is dependent on *CONSTANS (CO)* (Martinez-García *et al.*, 2002), the key player in the photoperiod-dependent pathway in flowering time control. Interestingly, transgenic approaches revealed that flowering time control in *Arabidopsis* is linked to CO-regulated conversion of 12-OH-JA to its inactive sulphated derivative (Gidda *et al.*, unpublished information) suggesting a common leaf-generated signal for both photoperiod-dependent morphogenic processes.

### 7.6.3 Flower formation

Clear evidence for the role of JA in flower formation came from identification of the JA-insensitive mutant *coi1* and the JA-deficient mutants *fad3-2fad7-2fad8*,

*dad1*, *opr3* and *dde1* (see Section 7.4) as well as from the AOS-knockout mutant (Park *et al.*, 2002) of *Arabidopsis*. Except for *dad1*, all of these mutants are male sterile. Interestingly, the *CO11* homologue of tomato affected in the JA-insensitive mutant *jail* is female sterile suggesting different JA-dependent processes in flower development between these species (Li *et al.*, 2004b). Further evidences for the role of JA in flower formation is given by distinct expression of JA-biosynthetic genes in flower organs, such as ovules correlating with elevated levels of jasmonates, and by the fact that flower organs exhibit a distinct oxylipin signature (Hause *et al.*, 2000). The ratio as well as total amount of oxylipins in different flower organs can be shifted by constitutive over-expression of the *AOC* (Miersch *et al.*, 2004).

The *AOC* expression and the elevated JA levels in ovules and pistils, respectively, of tomato flowers (Hause *et al.*, 2000) correlated with expression of defense genes, such as *PIN2* (Peña-Cortés *et al.*, 1991) or threonine deaminase (Samach *et al.*, 1995). Also flowering time control, well defined by a sequential and concerted activity of genes orchestrated in different pathways (Mouradov *et al.*, 2002), seems to be affected by jasmonates. The level of 12-OH-JA, regulated in a CO- and CO11-dependent manner, affects the onset of flowering (Gidda *et al.*, unpublished data). Although not clearly understood, mutant and expression analyses of JA-induced genes suggest a role for JA and related compounds in the following processes of flower formation and functions:

- (1) Anther development and dehiscence
- (2) Female organ development
- (3) Increased defense status of a sink tissue, such as ovules
- (4) emission of volatiles to attract insects for pollination
- (5) Formation of deterrent alkaloids and glucosinolates
- (6) Formation of secondary metabolites functioning in colour formation of petals.

An interesting new aspect on cross-talk between JA and auxin in flower maturation was found recently (Nagpal *et al.*, 2005). The auxin-response factors ARF6 and ARF8 were shown to promote JA formation leading to proper filament elongation and pollen release of anthers. Correspondingly, in the double mutant *arf6-2arf8-3* genes encoding JA biosynthesis enzymes are under-expressed (Nagpal *et al.*, 2005). This corresponds well to the phenotype of JA-deficient mutants (see Section 7.4). The role of JA in anther development is indicated by male sterility of JA-deficient mutants and by microarray analyses of developing anthers showing up-regulation of JA-inducible genes in stage 12 of flower development, when anthers become mature (Mandaokar *et al.*, 2003).

#### 7.6.4 Senescence

Senescence is the terminal phase in the development of plant organs, accompanied by a dramatic reprogramming of the anabolic and catabolic capacity. More than 100 genes, including those encoding nucleases, proteinases, lipases and chlorophyllases, are specifically up-regulated during leaf senescence (senescence associated genes,

SAGs) (Gan & Amasino, 1995). A large-scale screen for genes up-regulated in *Arabidopsis* in response to ABA, SA, JA or stress revealed a subset of JA-responsive genes containing many SAGs (He *et al.*, 2001). *SAG12* encoding a cysteine protease is a marker for the onset of senescence. *SAG101* was shown to code for an acyl hydrolase with a significant role in senescence (He *et al.*, 2001). Interestingly, *SAG101* is an essential component of plant innate immunity against biotrophic pathogens by interacting with ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4) (Feys *et al.*, 2005). During senescence endogenous content of JA is augmented, and expression of many JA-biosynthetic genes is up-regulated. Correspondingly, transgenic barley constitutively over-expressing the barley *LOX2* gene known to function in JA biosynthesis exhibited a senescent phenotype (Sharma *et al.*, 2006). Leaf senescence does not occur in the JA-insensitive mutant *coi1*, affected in an F-box protein (Table 7.1; He & Gan, 2002; He *et al.*, 2002), while mutation of another F-box protein, ORE9, resulted in delayed senescence (Woo *et al.*, 2001).

There is an antagonistic and synergistic overlap in the activity of genes related to photosynthesis, stress responses and senescence (Wasternack, 2004). Recent microarray analyses revealed which metabolic pathway of carbon partitioning and nitrogen remobilization is preferentially activated during senescence (Guo *et al.*, 2004; Lin & Wu, 2004; Buchanan-Wollaston *et al.*, 2005). Among the transcription factor gene families such as *WRKYs*, *NACs*, *bZIPs*, and *ERFs*, several members are associated with senescence. How coordinate activity of transcription factors may regulate senescence- and defense-related gene activity was shown for *WRKY6* (Robatzek & Somssich, 2001). Transcriptome analysis of senescent *Arabidopsis* leaves was recently combined with a metabolome analysis using early senescing and late senescing lines (Diaz *et al.*, 2005). Interestingly, Glu, Asp, Leu and Ile could be identified as chemical markers of the degree of senescence. It will be interesting to see how JA is linked to this metabolic reprogramming during leaf senescence. In case of chlorophyll degradation, the first visible symptom of senescence, the senescence-promoting effect of JA known from the 1980s, may occur by induction of chlorophyllase (Tsuchiya *et al.*, 1999).

## 7.7 Concluding remarks

In the past two decades an exponential increase in the number of publications appeared showing an impressive increase in knowledge on the formation and action of jasmonates. After elucidation of the biosynthetic pathway and several physiological effects in the 1980s, a shift to molecular-genetic analyses of biosynthesis, signaling and action of jasmonates appeared in the 1990s. A breakthrough was the identification of mutants affected in JA biosynthesis and signaling combined with transgenic approaches. These tools allowed questions on the function of JA in plant-microbe interactions and many other stress responses as well as its role in developmental programmes, such as flower development and leaf senescence to be

addressed. At the same time, it became clear that JA formed part of a signaling network involving other molecules, such as SA, ethylene, and ABA. New tools for the analyses of JA and related compounds of the LOX pathway, the oxylipins, led to a much broader perspective, leading to questions on which of the compounds is most active in a particular signaling pathway. Currently, analyses of the transcriptome, proteome and metabolome are improving our understanding of metabolic links and gene activities occurring in processes in which JA is involved.

Future work will address questions on JA and oxylipin perception, *cis*- and *trans*-acting factors regulating JA-responsive promoters, MAP kinases active in JA signaling, cross-talk of JA with other signals and diversity of action of JA in the plant–plant and plant–microbe interactions under natural conditions. Analyses of cell- and organ-specificity of action of jasmonates will improve our understanding on their role in developmental processes.

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## 8 Salicylic acid

Christophe Garcion and Jean-Pierre Métraux

### 8.1 Introduction

The initial interest in the salicylates is due to its medicinal virtues and can be traced to ancient times. In North America, Indians discovered the curing properties of willow tree bark while the Greek physician Hippocrates (400 BC) prescribed willow decoctions to ailing patients. There are several accounts, on the history of salicylic acid (SA) for the interested reader to consult (Raskin, 1992; Pierpoint, 1994; Rainsford, 2004).

Various physiological processes have been associated with SA and include flowering, thermogenesis, stomatal closure and leaf abscission (reviewed by Raskin, 1992). Exogenous SA was first shown in tobacco to protect against tobacco mosaic virus (TMV) and was found to be conspicuously associated with the induction of pathogen-related proteins (PRs) (White, 1979). Later, SA was found in plants after pathogen infection, locally and systemically, making SA an endogenous signal for systemic acquired resistance (SAR; reviewed in Sticher *et al.*, 1997). These observations opened the way for studies on the molecular mechanism of plant defence. It followed an unprecedented spurt of research activity on the action of SA in the general context of plant–pathogen interactions. This review is an attempt to provide a large overview of the current status of this research.

Strong correlations were found between induced resistance, endogenous SA accumulation and gene expression in plant tissue after a localized pathogen infection (reviewed in Sticher *et al.*, 1997). Further support for the importance of SA for SAR came from studies with mutants and transgenic plants that exhibit altered levels of SA. In general, plants with low endogenous SA are impaired in SAR. Conversely, mutants with constitutive high levels of SA exhibit increased tolerance to pathogens (reviewed in Métraux & Durner, 2004).

Later, other endogenous signaling molecules were associated with the SA-independent activation of resistance response. These compounds include octadecanoic acid derivatives such as jasmonic acid (JA), methyl jasmonate (MeJA) and 12-oxo-phytodienoic acid (OPDA), as well as ethylene (ET). Interestingly, in *Arabidopsis thaliana*, SA-dependent responses can provide resistance to a specific spectrum of pathogens, such as *Hyloperonospora parasitica* or *Pseudomonas syringae*, while JA- and ET-dependent resistance responses seem to operate against another group, for example *Alternaria brassicicola* and *Botrytis cinerea* (Thomma *et al.*, 1998). Thus, a pathogen attack does not trigger a central SA-dependent cascade of reactions leading to the activation of a single set of resistance mechanisms, but rather activates a complex network dependent on multiple signals, of which SA is one (Thomma *et al.*,

1998; 2001). Some branches of this network intercommunicate or cross-talk with each other, or interfere with pathways triggered by environmental stimuli, such as light (Genoud *et al.*, 2002). This increases the flexibility of the network to optimize the defensive reactions of the plant to a given environment. A digital approach based on Boolean logic was proposed to represent such a complex network (Genoud *et al.*, 2003).

This chapter will focus on our state of knowledge on the biosynthesis and metabolism of SA, SA-dependent signaling and its mode of action.

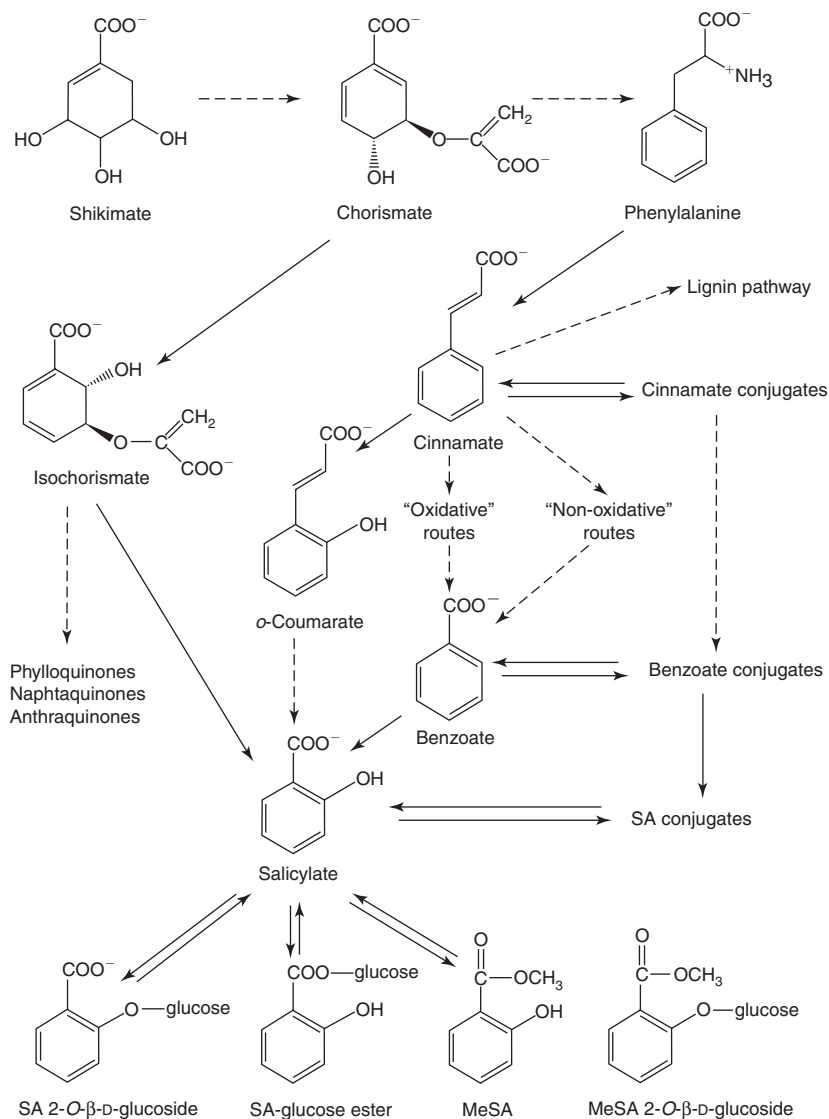
## 8.2 Biosynthesis and metabolism of SA

### 8.2.1 SA biosynthesis via the phenylpropanoid pathway

Studies using radiolabelled compounds in the early 1960s indicate that in higher plants SA derives from the phenylpropanoid pathway (see review by Lee *et al.*, 1995). In the first step of this process, trans-cinnamate (tCA) is produced by deamination of L-phenylalanine, in a reaction catalysed by the phenylalanine-ammonia lyase (PAL) enzyme. Two routes, each comprising a side-chain shortening reaction and an *o*-hydroxylation reaction, were found to convert tCA into SA. tCA could be either hydroxylated to *o*-coumarate before decarboxylation to SA, or it could be converted first to benzoic acid (BA), then hydroxylated into SA (Fig. 8.1). The *o*-coumarate pathway was established only from few reports (see review by Lee *et al.*, 1995), and no supporting evidence could be found for this pathway in tobacco (Yalpani *et al.*, 1993) or rice (Silverman *et al.*, 1995).

More recent studies brought further evidence to support the BA pathway. The role of PAL in SA biosynthesis was first investigated by examining if both the substrate and the reaction product for this enzyme could be precursors for SA. Indeed, in cucumber and in potato,  $^{14}\text{C}$ -phenylalanine can be metabolized into radiolabelled SA (Meuwly *et al.*, 1995; Coquoz *et al.*, 1998). Applied labelled tCA is converted into SA in cucumber, rice, potato and tobacco (Meuwly *et al.*, 1995; Silverman *et al.*, 1995; Coquoz *et al.*, 1998; Chong *et al.*, 2001). The role of PAL in SA biosynthesis was further demonstrated using the specific inhibitor 2-aminoindan-2-phosphonic acid (AIP; Zon & Amrhein, 1992). In cucumber and in potato, inactivation of PAL by AIP prevents the conversion of  $^{14}\text{C}$ -phenylalanine into SA, but not the synthesis of SA from tCA or BA (Meuwly *et al.*, 1995; Coquoz *et al.*, 1998). In *Arabidopsis*, AIP treatment suppresses the rise of SA levels after infection. Resistant plants become susceptible to an avirulent strain of *Hyloperonospora* after AIP treatment, but supplying SA restores the resistance (Mauch-Mani & Slusarenko, 1996).

The conversion of  $^{14}\text{C}$ -tCA into SA via BA in cultured tobacco supports the involvement of BA in the biosynthesis of SA (Yalpani *et al.*, 1993). The generation of BA from tCA was clearly established in potato (Coquoz *et al.*, 1998), but was not detected in cucumber (Meuwly *et al.*, 1995) and BA was also demonstrated to be a possible SA precursor in other plants, including cucumber and rice (Klämbt,



**Figure 8.1** Pathways of SA biosynthesis and metabolism.

1962; Meuwly *et al.*, 1995; Silverman *et al.*, 1995; Coquoz *et al.*, 1998; Ogawa *et al.*, 2005). However, the intermediacy of free BA in SA biosynthesis was questioned in tobacco in a later study (Chong *et al.*, 2001). This work relied for the first time on a specific method to determine BA levels, and on the use of piperonylic acid to block cinnamate-4-hydroxylase and the downstream lignin pathway, therefore



redirecting tCA into the SA pathway. On the basis of radioisotope dilution experiments, Chong and colleagues proposed that conjugated forms of BA, rather than free BA, participate in SA biosynthesis in tobacco. However, whether free BA or conjugated forms of BA are involved is still not completely resolved as the results from radioisotope dilution experiments could also be partly explained by metabolic channelling, which has been shown to occur in the phenylpropanoid pathway (Winkel, 2004; Jorgensen *et al.*, 2005).

Two enzymatic pathways have been proposed to bring about side-chain shortening reaction of tCA to BA: the CoA-dependent, oxidative route, through 3-hydroxy-3-phenylpropanoate-CoA, which is similar to fatty acid  $\beta$ -oxidation, and the (incorrectly named) "non-oxidative", CoA-independent route through benzaldehyde (see available evidence and references in Lee *et al.*, 1995). Recent work showed that benzaldehyde is unlikely to be an intermediate in SA biosynthesis (Ribnicky *et al.*, 1998), whereas radiolabelled 3-hydroxy-3-phenylpropanoic acid is metabolized into SA in tobacco and cucumber, and produced in these species from phenylalanine (Jarvis *et al.*, 2000). The decarboxylation of tCA into BA in the course of SA biosynthesis in tobacco appears to follow the oxidative route, but the enzymes involved in this reaction still remain elusive.

However, as emphasized in several reports, the non-oxidative route could also participate in the side-chain shortening from tCA to BA, independently of the SA production. BA biosynthesis in cell cultures of *Hypericum androsaemum* might proceed through a combination of the "oxidative" and "non-oxidative" pathways, but no evidence from experiments with radiolabelled compounds was presented (Abd El-Mawla & Beerhues, 2002). In petunia petals, the chemical network between tCA and BA was investigated using deuterium-labelled phenylalanine (Boatright *et al.*, 2004). In this system, both "oxidative" and "non-oxidative" pathways were active. Based on a model of the fluxes between benzenoid compounds, benzylbenzoate was predicted to be an intermediate between phenylalanine and BA (Boatright *et al.*, 2004). In *Astilbe chinensis*, the BA-synthesizing activity was localized in chloroplast fractions (Löffelhardt & Kindl, 1975). A similar localization was observed in *Nasturtium officinale* and *Hydrangea macrophylla*. In these experiments, labelled phenylalanine was more effectively converted than CA into BA, possibly indicating the existence of metabolic channelling (Löffelhardt & Kindl, 1975). However, these experiments were performed in conditions where the SA biosynthesis pathway is presumably not induced, or only induced at basal levels. At the moment it remains unknown if the BA-producing reactions described above could be involved in the production of SA following pathogen infection.

Whether free or conjugated forms of BA are the major intermediates in SA biosynthesis, a BA 2-hydroxylase (BA2H) is likely to catalyse the final step leading to SA in the phenylpropanoid pathway. BA2H, which appears to be an oxygenase belonging to the cytochrome P450 family was partially purified in tobacco and shown to be strongly induced after inoculation with TMV (Leon *et al.*, 1995). BA2H activity was also detected in rice (Silverman *et al.*, 1995).

### 8.2.2 SA biosynthesis through the isochorismate pathway

SA is synthesized and used as a siderophore precursor in different bacteria genera such as *Pseudomonas*, *Burkholderia*, *Azospirillum*, *Vibrio*, *Yersinia* and *Mycobacterium* (see references in Gaille *et al.*, 2002). The biosynthesis of SA was recently established in *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Yersinia enterocolitica* and *Mycobacterium tuberculosis*, and was shown to branch from chorismate. In *P. aeruginosa* and *P. fluorescens*, SA is produced in two distinct steps: chorismate is first isomerized into isochorismate by an isochorismate synthase (ICS – also called isochorismate hydroxymutase), followed by an isochorismate pyruvate lyase (IPL)-catalysed conversion of isochorismate into SA and pyruvate (Mercado-Blanco *et al.*, 2001; Gaille *et al.*, 2002; Gaille *et al.*, 2003). In *Y. enterocolitica* and *M. tuberculosis*, SA is produced from a single bifunctional salicylate synthase that also proceeds via isochorismate (Kerbarh *et al.*, 2005b; Zwahlen *et al.*, 2005).

Genetic evidence indicates that most SA is synthesized in *Arabidopsis* by a similar pathway as in bacteria (Wildermuth *et al.*, 2001). Two independent mutants of the *ICS1* gene were found to accumulate low levels of SA after pathogen infection (5–10% of the wildtype; Nawrath & Métraux, 1999; Wildermuth *et al.*, 2001). The *ICS1* gene product shares 57% amino acid sequence identity with an ICS isolated from *Catharanthus roseus*, the activity of which was confirmed biochemically. *ICS* genes have been detected in other species, including soya bean, tomato, tobacco and medicinal plants (van Tegelen *et al.*, 1999a & b; Wildermuth *et al.*, 2001; Ogawa *et al.*, 2005). ICS activity was detected, but not associated with SA biosynthesis, in cell cultures from the *Rubiaceae* family (Leduc *et al.*, 1991; Poulsen *et al.*, 1991). Interestingly, the ICS enzyme may be located in plastids, as suggested by the predicted plastid targeting sequence encoded in the *ICS* genes, but this has not been definitely demonstrated. This localization agrees with a commonly held hypothesis that chorismate is produced and mainly available in plastids. Indeed, when a chimeric SA synthase composed of fused bacterial ICS and IPL is expressed in *Arabidopsis*, SA levels are much higher when the hybrid enzyme is targeted to plastids rather than to the cytosol (Mauch *et al.*, 2001). A later study in tobacco showed a large amount of chorismate available in plastids (Viitanen *et al.*, 2004). So far, no IPL activity has been reported in plants.

The ICS enzyme is not specific to SA biosynthesis. In higher plants, as in bacteria, isochorismate can be metabolized into various compounds, including phyloquinones, anthraquinones and dihydroxybenzoic acid (Leistner, 1999; Muljono *et al.*, 2002). In a recent work on the *acd11* mutant of *Arabidopsis*, exogenous SA could not compensate the effects of a mutation in the *ICS1* gene. The authors proposed that isochorismate-derived compounds other than SA were necessary for the *acd11* phenotype (Brodersen *et al.*, 2005).

Studies of the isochorismate pathway in plants could benefit from the strong interest raised in bacterial SA synthases that are envisioned as potential drug targets in medicine, and present peculiar enzymatic properties. Several studies recently focused on protein crystallization and development of inhibitors (Kozlowski *et al.*,

1995; DeClue *et al.*, 2005; Harrison *et al.*, 2005; Kerbarh *et al.*, 2005a; Kunzler *et al.*, 2005; Payne *et al.*, 2005).

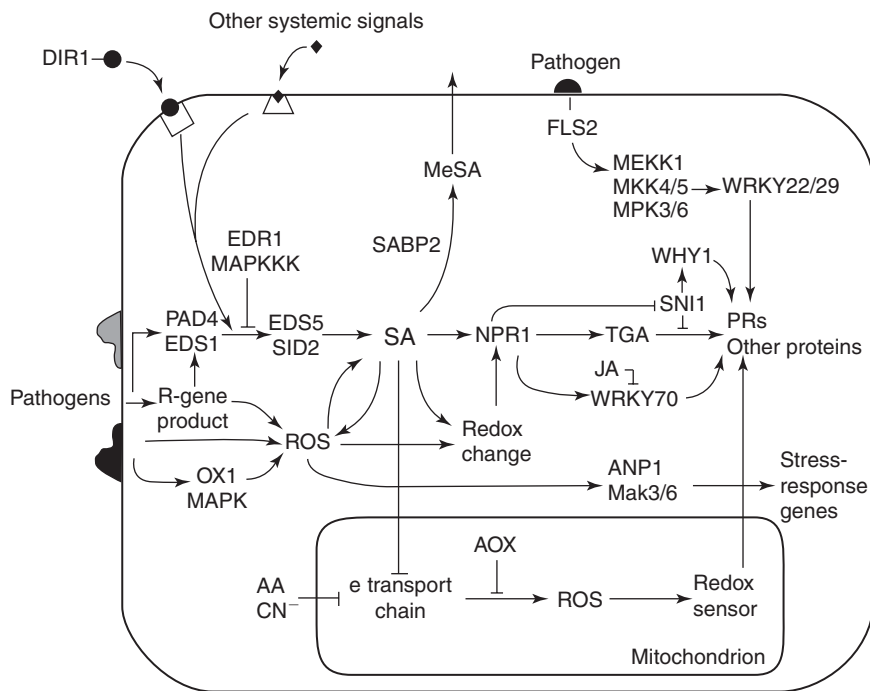
### 8.2.3 *Relative contribution of the isochorismate and BA pathway*

The unexpected discovery of the isochorismate pathway in *Arabidopsis* does not invalidate the results obtained from studies on the BA pathway. Obviously, identification of two pathways for SA biosynthesis in plants poses the question of their co-existence in the same organism/tissue/cell, and their relative contributions. Studies relying on tracer compounds were hampered by the incorporation of most of the radioactive compounds into the phenylpropanoid and lignin pathways, resulting in low amounts of labelled SA. However, as emphasized by Wildermuth *et al.* (2001), lower than expected specific radioactivity values have been obtained in experiments, indicating the existence of another pathway. Furthermore, inhibition of PAL by AIP reduced, but did not abolish the induction of SA (Mauch-Mani & Slusarenko, 1996; Coquoz *et al.*, 1998). This result could be explained either by incomplete inhibition of PAL by AIP, or by the activity of the isochorismate pathway.

The relative contribution of each pathway may well differ between species. The two pathways might also operate under different conditions, although this is unlikely in *Arabidopsis*, as an *ics1* mutant failed to respond to various SA-inducing conditions (Nawrath & Métraux, 1999). For instance, in the case of ozone exposure, a recent study showed that while in tobacco radioactive BA is incorporated into SA, no ICS activity could be detected and the unique *ICS* gene in the genome is not expressed. In contrast, in *Arabidopsis* the same conditions lead to the expression of the *ICS1* gene and detectable ICS activity (Ogawa *et al.*, 2005).

### 8.2.4 *Regulation and localization of SA biosynthesis*

In plants with low basal levels of free and total SA, its biosynthesis may be induced by biotic and abiotic stresses. For instance, in *Arabidopsis* and tobacco, SA accumulation is triggered by UV-C radiation or ozone exposure (reviewed in Métraux & Durner, 2004). In plants which possess a high basal level of SA, such as rice and potato, pathogen inoculation may not necessarily induce *de novo* SA biosynthesis (Silverman *et al.*, 1995), but application of arachidonic acid on potato leaves can induce SA formation (Coquoz *et al.*, 1995). Upstream regulators of the activation of the SA biosynthesis pathway have been identified in genetic screens and include EDS1, PAD4, NDR1 and EDS5. EDS1 and PAD4 are two proteins of unknown function containing a lipase domain that are essential for the resistance to *Pseudomonas syringae* and *Peronospora parasitica*. These proteins are likely to be close to the perception step of the pathogens (reviewed in Durrant & Dong, 2004; Métraux & Durner, 2004). The regulation of SA accumulation might require an interaction of EDS1 with PAD4. EDS1 is necessary for the transcriptional regulation of PAD4 while both proteins are necessary for the expression of EDS5, a putative MATE transporter involved in the control of SA accumulation after pathogen attack and exposure to UV-C light



**Figure 8.2** Overview of SA-signaling pathways.

(Nawrath & Métraux, 1999). The expression of EDS1 and PAD4 can also be up-regulated by SA and a positive feedback loop was postulated to amplify the SA pathway (reviewed in Shah, 2003).

From the available evidence, SA is produced in high amounts at/around infection sites or at the site of the hypersensitive reaction (HR), a form of programmed cell death response induced by plants in response to avirulent pathogens (Enyedi *et al.*, 1992). Furthermore, SA was found in the phloem and in non-infected upper leaves of plants with localized infections on the lower leaves (Sticher *et al.*, 1997). A more precise tissue localization is not available at this point.

### 8.2.5 Metabolism of SA

Many plant species produce various compounds derived from SA (reviewed by Pierpoint, 1994). The chemical fate and the metabolism of SA were probed by feeding radioactive SA to various plant tissues. For example, SA is transformed into gentisic acid (2,5-dihydroxybenzoic acid) and *O*-pyrocatechuic acid (2,3-dihydroxybenzoic acid), bound to glycosides or eventually esterified into methylsalicylate (MeSA) (see references in Lee & Raskin, 1998). In tomato, gentisic acid was found to be produced from SA after viral infections, and to be associated with the

induction of a set of PRs different from that induced by SA. Gentisic acid was proposed to act as a pathogen-induced signal in addition to SA (Belles *et al.*, 1999). However, glycosylation was the most frequently reported modification of SA.

In healthy or infected tobacco, the main compounds derived from SA are SA 2-*O*- $\beta$ -D-glucoside (SAG) and the glucosyl salicylate ester (GSE) (Fig. 8.1), with the latter occurring transiently and in lower quantities than the former (Edwards, 1994; Lee & Raskin, 1998). In some cases MeSA and its derived glucose conjugate could also be detected (see below; Shulaev *et al.*, 1997; Dean *et al.*, 2005). The conversion of SA into SAG was also observed in rice and in soya bean (Silverman *et al.*, 1995; Dean *et al.*, 2003). Endogenous SA is metabolized in a similar manner in all species studied so far (see references in Lee *et al.*, 1995).

Glycosylation of SA is postulated to prevent the accumulation of toxic levels of free SA, and thus to regulate SA signaling. Such roles have been well established in the case of several compounds (see for instance the review by Gachon *et al.*, 2005). SAG in soya bean and tobacco is mainly produced in the cytosol (Dean *et al.*, 2003; 2005) and transported into the vacuole, either via a tonoplast-localized ABC transporter in soya bean (Dean & Mills, 2004), or an H<sup>+</sup>-antiporter in tobacco (Dean *et al.*, 2005). SAG has also been proposed to act as a storage form of SA since exogenously applied SAG is inactive and can be converted back into SA (Hennig *et al.*, 1993; Kawano *et al.*, 2004). Release of SA from exogenous SAG may occur via the enzymatic activity of a  $\beta$ -glucosidase (Seo *et al.*, 1995), but to our knowledge it is still not clear if endogenous SAG, once stored in the vacuole, can be mobilized back into SA. Storage of SA precursors was also suggested in the form of conjugated BA, however accumulation of this compound could not be confirmed in a later study (see Chong *et al.*, 2001).

An UDP-glucose SA glucosyltransferase (SAGT) catalysing the formation of both SAG and GSE was purified and cloned in tobacco (Lee & Raskin, 1999). The enzyme was not specific to SA but could accept several phenolics as substrates. In *Arabidopsis*, a study focused on the glycosylation of benzoates identified two enzymes that could glycosylate SA (Lim *et al.*, 2002). The SAGT activity in soya bean, rice and tobacco was induced by SA (Silverman *et al.*, 1995; Lee & Raskin, 1999; Dean *et al.*, 2003). In tobacco, inoculation with TMV or with *P. syringae* pv. *phaseolicola* also resulted in the induction of the cloned *SAGT* gene (Lee & Raskin, 1999). Loss-of-function experiments for *SAGT* genes either in tobacco or in *Arabidopsis* would provide valuable information on the roles of SAG and GSE, but have not yet been reported.

In plants, the only information available on SA turnover comes from a study by Edwards (1994). In his work, [7-<sup>14</sup>C]SA fed to tobacco leaf disks resulted in emission of <sup>14</sup>C-CO<sub>2</sub>, suggesting that SA, or more likely its conjugates, could be decarboxylated.

### 8.2.6 Biosynthesis of MeSA

The volatile MeSA (Fig. 8.1) is a component of the flavour of fruits such as plum, strawberry, black cherry and tomato, of the floral scents of many plant species, and

occurs in vegetative tissues of several species including tobacco, strawberry, fig, oat and *Arabidopsis* (see references in the review of Seskar *et al.*, 1998; Van Poecke *et al.*, 2001; Effmert *et al.*, 2005). MeSA emission from flowers was linked with the attraction of moth pollinators, and MeSA also has been proposed to act as an airborne defence signal when released from vegetative tissues (Shulaev *et al.*, 1997).

Early reports established that MeSA was present in *Gaulthiera procumbens* and was formed from CA, suggesting common biosynthesis steps with SA (reviewed in Verberne *et al.*, 1999). MeSA is synthesized from SA in tobacco (Shulaev *et al.*, 1997; Dean *et al.*, 2005). The enzyme that catalyses this reaction, an SA carboxyl methyl transferase, has been cloned in several species, including *Arabidopsis*, *Clarkia breweri*, *Stephanotis floribunda* and snapdragon (see Chen *et al.*, 2003 and references therein). In *Arabidopsis*, expression of the corresponding gene was found in flowers, although no emission of MeSA was detected from this tissue, and in trichomes and hydathodes of leaves. The enzyme is induced by alamethicin (a fungal antibiotic), *Plutella xylostella* herbivory, physical wounding and MeJA.

MeSA does not appear to have an effect *per se*, but must be first converted back into SA to act as a signaling factor (Shulaev *et al.*, 1997; Seskar *et al.*, 1998). A MeSA esterase was found in tobacco and shown to be a SA-binding protein (SABP2; see Section 8.3.1; Forouhar *et al.*, 2005). Glycosylation of MeSA into MeSA 2-*O*- $\beta$ -D-glucose (Fig. 8.1) occurs in tobacco cell cultures, but this metabolite was not detected in tobacco leaves, possibly reflecting differences between cell cultures and intact leaves, or between various ecotypes (Dean *et al.*, 2005).

### 8.3 Signal transduction and mode of action

Ectopically applied SA, along with BA and aspirin, was first described to induce resistance to TMV and PR proteins in tobacco (White, 1979). After inoculation by pathogens, the level of SA increases *in planta* and SA was proposed to be a signal for SAR. This initial hypothesis has been subsequently supported by abundant genetic evidence. Plants impaired in the accumulation of normal SA levels are affected in their resistance to pathogens. For instance, over-expression of a bacterial gene encoding a SA hydroxylase (the *NahG* gene) that converts SA to catechol, or mutants with low levels of SA, are unable to mount SAR (Delaney *et al.*, 1994). In contrast, enhanced pathogen resistance is observed in transgenic plants expressing genes for the biosynthesis of SA or mutants that hyper-accumulate SA (Verberne *et al.*, 2000; Mauch *et al.*, 2001; Métraux & Durner, 2004). All these observations strongly support a role for SA as an endogenous signal in the transduction of the resistance response to pathogens. Functional analogues of SA such as BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; BION<sup>®</sup>, ACTIGARD<sup>®</sup>) and INA (2,6-dichloroisonicotinic acid) also induce pathogen resistance and the accumulation of PR-proteins (Métraux *et al.*, 1991). These compounds do not act by inducing an increase in SA but seem to have a similar mode of action on the plant. They provided a proof of concept for the development of a new generation of plant protectants.



### 8.3.1 SA-binding sites

Soon after its discovery SA was accepted as a hormone, and the conceptual framework used in hormone research was employed to study and understand its molecular action. An important entry point in this research was the determination of a potential receptor for SA. Binding studies in tobacco using radiolabelled SA identified catalase as the first SABP. Catalase is inactivated upon binding SA ( $K_d$  of 14  $\mu\text{M}$ ) resulting in an increase in intracellular  $\text{H}_2\text{O}_2$ . This was proposed to activate defence gene expression or act as an antimicrobial barrier at the site of invasion (Chen *et al.*, 1993).

SA, as well as the functional analogue INA are inhibitors of ascorbate peroxidase (APX), another  $\text{H}_2\text{O}_2$ -scavenging enzyme. The inhibition of both catalase and APX, but not of guaiacol peroxidases, which are involved in cell-wall biosynthesis supports a model whereby SA-induced defence responses are mediated through elevated levels of  $\text{H}_2\text{O}_2$  or coupled to perturbations of the cellular redox state (Durner & Klessig, 1995). However, the hypothesis that SA acts *via* inhibition of catalase was questioned for several reasons and  $\text{H}_2\text{O}_2$  was placed upstream of SA in the signal transduction pathway (reviewed in Mauch-Mani & Métraux, 1998).

Another SABP (SABP2) was then found in tobacco that exhibits high affinity for SA ( $K_d$  of 90 nM) (Du & Klessig, 1997). This SABP2 has an esterase and an SA-inducible lipase activity (Kumar & Klessig, 2003). Silencing of SABP2 using RNA interference leads to a loss in local and systemic-induced resistance to TMV in tobacco (Kumar & Klessig, 2003). SABP2 was assigned to the  $\alpha/\beta$  hydrolases superfamily by 3D analysis of its crystalline structure. This method also confirmed the binding of MeSA to the active catalytic Ser-His-Asp motif of SABP2. Since MeSA is the substrate with the highest binding activity for SABP2 and needs to be converted to SA for activity (Seskar *et al.*, 1998), SABP2 may participate in the control of intracellular SA levels rather than being a true SA receptor (Forouhar *et al.*, 2005) (Fig. 8.2).

A further SABP, SABP3, localized in the soluble fraction of purified tobacco leaf chloroplasts, was identified as carbonic anhydrase (CAN) (Slaymaker *et al.*, 2002). SA binds CAN with moderate affinity ( $K_d$  of 3.7  $\mu\text{M}$ ) and the enzymatic activity of CAN is unaffected by SA binding. Besides the carbon fixing activity, CAN has antioxidant properties. A recombinant SABP3/CAN as well as a close homologue of SABP2 from tobacco complements a yeast mutant sensitive to oxidative stress. Silencing of the CAN in *Nicotiana benthamiana* suppressed the hypersensitive response in a race-specific plant-pathogen interaction. Thus, three proteins interacting with SA have antioxidant activities. Since SA inhibits both CAT and APX, it has been speculated that SA may modify the antioxidant activity of CAN. As proposed tentatively by Slaymaker *et al.* (2002), the ability of SA to inhibit both cytosolic and chloroplast antioxidant enzymes might be part of its molecular action. By increasing the level of reactive oxygen species (ROS), SA may activate a positive feedback loop for SA production and thus induce defence responses following pathogen infection (Slaymaker *et al.*, 2002).

SA is able to form free radicals upon inhibition of heme-containing enzymes, such as peroxidase or catalase. This led to the “free radical” hypothesis of SA action (Durner & Klessig, 1995; Durner & Klessig, 1996), in which SA action is related to



the effect of free radicals on lipid peroxidation, the products of which might activate defence reactions (Farmer *et al.*, 1998). The quantitative and topological coincidence of free radicals with the induction of resistance needs yet to be fully assessed.

### 8.3.2 SA and signal transduction mediated by MAP kinases

Many studies on the action of SA have been directed at the reactions downstream of a potential interaction with a binding site. In eukaryotes, protein kinases belonging to the MAP kinase (MAPK) family are activated by numerous biotic or abiotic stimuli and transduce external stimuli into endogenous responses (Widmann *et al.*, 1999). Usually, the MAPK signaling involves a sequential and reversible phosphorylation of MAPK by MAPK kinases (MAPKK), themselves phosphorylated by MAPKK kinases (MAPKKK). The *Arabidopsis* genome includes 20 MAPKs, 10 MAPK kinases and 60 MAPK kinase kinases (Ichimura *et al.*, 2002) that define major signaling pathways (Nakagami *et al.*, 2005). The components of the MAPK pathway can operate in different combinations and depend on the biological context. This explains part of the exquisite specificity of cellular responses and adds tremendous complexity to the deciphering of the MAPK signaling. SA and its biologically active analogues induce the activity of a protein kinase in tobacco (SA-induced proteinase kinase or SIPK) belonging to the MAPK family (Zhang & Klessig, 1997). A wound-induced MAPK (WIPK) is activated in tobacco upon inoculation with TMV (Zhang & Klessig, 1998). WIPK is not activated in tobacco lacking the TMV-resistance gene *N*. Activation of WIPK by TMV is SA independent and WIPK was placed upstream of SA in the signal transduction pathway leading to the hypersensitive cell death (HR) defence reaction (Zhang & Klessig, 1998). Both SIPK and WIPK are activated by NtMEK2, an MAPKK, and expression of constitutively active mutants of *NtMEK2* induces the HR and activation of the expression of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and *PAL* genes of the biosynthetic pathway of phenolics related to defence (Yang *et al.*, 2001). Further support for the involvement of this signaling cascade was provided by virus-induced gene silencing (VIGS) of *MEK2*, *SIPK* or *WIPK*. Suppression of these three genes attenuated the *N* gene-mediated resistance to TMV (Jin *et al.*, 2003). There exists another MAPK pathway involved in the architecture of the phragmoplast at the late M phase of the cell cycle. It involves the MAPKKK NPK1 and its respective downstream partners, the MAPKK NQK1/NtMEK1 and the MAPK NRK1 (Soyano *et al.*, 2003). Silencing of *NPK1*, *MEK1* and *NTF6/NRK1* interfered with the activity of the tobacco resistance gene *N* (Liu *et al.*, 2004). The search for events activated downstream of SIPK/WIPK in tobacco resulted in the identification of WRKY transcription factors as intermediates in the activation of defence genes (Kim & Zhang, 2004). Members of the WRKY superfamily of transcription factors are regulators of the plant–pathogen defence programme (Eulgem *et al.*, 2000; Maleck *et al.*, 2000). The WRKY proteins are defined by the conserved amino acid sequence WRKYGQK at the N-terminal end, and zinc-finger-like motif (Eulgem *et al.*, 2000). VIGS-induced down-regulation of the WRKY1–WRKY3 and MYB1 transcription factors also compromised N-mediated resistance (Liu *et al.*, 2004). Recently, SIPK was found to phosphorylate WRKY1 and to increase its binding to a W-box sequence

from the tobacco chitinase gene CHN50. Co-expression of SIPK and WRKY1 in *N. benthamiana* induces a stronger HR-like response than expression of SIPK alone. This points to WRKY1 as an important target downstream of the MAPK cascade in the activation of defence (Menke *et al.*, 2005).

Focusing on *Arabidopsis*, a complete MAP kinase cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) together with WRKY22/WRKY29 factors was found to operate upon activation of FLS2, a receptor for the bacterial elicitor flagellin (Fig. 8.2). This MAPK cascade mediates resistance to both *P. syringae* and *B. cinerea*, without the involvement of SA. The fungal and bacterial signals perceived by the plant are likely to be different, but converge at a conserved MAPK cascade (Asai *et al.*, 2002). The *Arabidopsis* AtMAPK4 is activated rapidly after wounding and regulates positively the JA-dependent but not the SA-dependent signaling pathway (Petersen *et al.*, 2000).

H<sub>2</sub>O<sub>2</sub> and other ROS are produced upon pathogen attack (Lamb & Dixon, 1997; Apel & Hirt, 2004). H<sub>2</sub>O<sub>2</sub> activates the MAPKKK ANP1 initiating phosphorylation of the SIPK analogues, AtMPK3 and AtMPK6, apparently without the involvement of SA (Kovtun *et al.*, 2000) (Fig. 8.2). The protein kinase OXI1 mediates ROS- and pathogen-induced activation of the *Arabidopsis* MPK3/MPK6 pathway (Rentel *et al.*, 2004); and a mutation in the *OXI1* gene strongly interferes with the activation of MPK3/MPK6 mediated by ROS and elicitors. The *ox1* mutants are hypersensitive to infection by virulent, but not avirulent strains of *H. parasitica*. This is reminiscent of tobacco, where the MEK2 pathway is upstream of the respiratory burst homologues A and B required for ROS production in response to infection (Yoshioka *et al.*, 2003). These observations indicate the existence of an amplification loop involving an MAPK cascade in the regulation of pathogen-induced ROS.

The *edr1* mutant shows increased resistance to powdery mildew and *P. syringae* related to SA-dependent defence responses (Frye *et al.*, 2001). The mutation was found in an MAPKKK similar to CTR1, a negative regulator of the ET response. EDR1 is likely to be upstream of an MAP kinase cascade that negatively regulates SA-inducible defence responses. Over-expression of a kinase-deficient full-length *EDR1* gene caused a dominant negative phenotype, with associated resistance to powdery mildew and enhancing ET-induced senescence (Tang & Innes, 2002). The position of EDR1 at the top of an MAPK cascade needs now to be confirmed. Interestingly, EDR1 may also regulate SA-dependent but not ET-dependent plant growth in response to drought stress (Tang *et al.*, 2005).

Summarizing, the follow-up of the initial observations on SA-induction of MAPK led to a wealth of data on the involvement of MAPK cascades in the pathogen-induced defence activation. How SA regulates MAPK signaling remains to be precisely determined.

### 8.3.3 SA and the central role of NPR1

The molecular action of SA was extensively investigated using genetic approaches. A number of reviews describe the signaling network of SA-induced resistance

(Glazebrook, 2001; Métraux & Durner, 2004; Durrant & Dong, 2004; Nawrath *et al.*, 2005). A pivotal protein in the transduction pathway from SA to the induction of PRs and defence responses is the ankyrin repeat-containing protein NPR1 (non-expressor of PR1) NIM1 (non-immunity) (Cao *et al.*, 1997; Ryals *et al.*, 1997; Dong, 2004; Pieterse & Van Loon, 2004) (Fig. 8.2). NPR1 function is essential for the induction of resistance by pathogens, by chemical inducers or by root-colonizing rhizobacteria (Pieterse *et al.*, 1998; Iavicoli *et al.*, 2003), as well as for priming (Conrath *et al.*, 2002). Genetic screens have been used to find possible intermediates linking SA to NPR1, but only alleles of *npr1* were reported (Glazebrook *et al.*, 1996; Shah *et al.*, 1997). Possibly, SA regulates NPR1 directly or mutations in other regulatory proteins might be lethal. These findings prompted further studies on the characterization of NPR1.

Over-expression of NPR1 leads to enhanced resistance to *P. syringae* and *H. parasitica* and to enhanced effectiveness of fungicides; this is of practical interest for a combined use of transgenic and chemical approaches for plant protection (Friedrich *et al.*, 2001). Induction of SA production in *Arabidopsis* leads to nuclear localization of NPR1 (Kinkema *et al.*, 2000), where it induces the expression of PRs *via* transcription factors. For instance, WRKY6 is associated with SA-induced expression of PR1, most likely *via* NPR1 (Robatzek & Somssich, 2002), and is autoregulated negatively. Similarly, AtWRKY18 positively regulates SA-induced and NPR1-dependent PR1 expression (Chen & Chen, 2002) and can be negatively regulated by other WRKY proteins. TGA transcription factors have also been found to be involved in NPR1-dependent activation of PRs (Zhang *et al.*, 1999; Despres *et al.*, 2000; Kim & Delaney, 2002). TGA transcription factors are characterized by a basic domain/Leu zipper (bZIP) sequence; their name refers to the TGACG-containing cognate DNA-binding element.

Using immunodetection of NPR1–GFP complexes in extracts of *Arabidopsis*, NPR1 was found to occur in an inactive state as an oligomeric complex. Upon the oxidative burst, associated with the induction of resistance by the chemical inducer INA, the monomeric form appears and is accompanied by the induction of PRs. The transition from the oligomeric to the monomeric form is regulated by the reduction of two conserved cystein residues, as indicated by mutational analyses. In its reduced form, NPR1 remains in the nucleus and PRs are induced constitutively (Mou *et al.*, 2003). This agrees with previous observations on the nuclear localization of NPR1 (Kinkema *et al.*, 2000) and demonstrates that the active form of NPR1 for PR-induction is monomeric and the control of the oligomeric to monomeric form is critical for the nuclear localization of NPR1. The redox status of the cell, as determined by the ratio of reduced/oxidized glutathione, indicates an initial oxidative burst after INA treatment that returns to a reduced state. Thus, the redox environment of NPR1 is likely to be the switch to activate the expression of PRs. The question has been raised of whether there are physiologically relevant levels of NPR1 oligomer in the cell (Somssich, 2003).

The importance of the regulation of the redox state of the cell for pathogen defence, stress responses, photosynthesis, hormonal responses, or growth and

development has been discussed in several reviews (Lamb & Dixon, 1997; Apel & Hirt, 2004; Mittler *et al.*, 2004; Torres & Dangl, 2005). However, it remains unclear how the reduced state is recovered after the oxidative burst. Possibly, SA may induce expression of genes encoding anti-oxidative proteins. One possible candidate is glucose-6-phosphate 1-dehydrogenase (G6PDH) of the pentose pathway which regulates cellular reducing power (Dong, 2004). Indeed, inhibition of G6PDH by 6-amino nicotinamide brings about a decreased reducing state, NPR1-monomer formation and *PR*-gene expression following SAR-induction (Mou *et al.*, 2003).

Although the oxidized form of TGA1 does not interact with NPR1 in a yeast two-hybrid screen, TGA1 or TGA4 interact with NPR1 in plant cells upon SA treatment. The normally oxidized cysteine residues in uninduced cells form intramolecular disulphide bonds, and after SA treatment, the cysteine residues are reduced and allow interaction with NPR1 (Despres *et al.*, 2003). Providing that TGA1 and TGA4 form part of the signaling cascade for SA-induced *PR* expression, these results strongly suggest that SA-induced *PR*-gene expression takes place under reducing conditions. The involvement of orthologues of the *Arabidopsis* TGA in the induction of race-specific resistance in tomato indicates that these signaling components are conserved in plants (Ekengren *et al.*, 2003). Thioredoxins and glutaredoxins are possible candidates for regulators of the redox state of NPR1 and TGA1; they are involved in the control of the cellular redox status (Gelhay *et al.*, 2005) and are well represented in the *Arabidopsis* genome. Further work is needed to verify this hypothesis and to clarify the details of thioredoxin-mediated control of NPR1 and TGA. In the first report providing evidence for the involvement of a plant thioredoxin in the regulation of disease resistance, VIGS or over-expression of the tomato thioredoxin CITRX was found to interfere with the establishment of the HR, induction of *PR*s and resistance of tomato to the fungal pathogen *Cladosporium fulvum* (Rivas *et al.*, 2004).

The cytoplasmic-located NPR1 mediates the cross-talk between the SA and the JA and ET-signaling pathways (Spoel *et al.*, 2003). The cytoplasmic form of NPR1 might regulate JA-induced gene expression by interfering with the ubiquitination and proteasome-mediated degradation of a putative negative regulator of JA signaling (Spoel *et al.*, 2003). An interesting role was found for the *Arabidopsis* WRKY70, a component downstream of NPR1 and common to both SA- and JA-mediated signaling pathways, which is activated by SA, but repressed by JA. Plants over-expressing *WRKY70* exhibit constitutive expression of SA-induced *PR* genes and of resistance to virulent pathogens, while in *WRKY70*-antisense-suppressed plants JA-responsive/COI1-dependent genes are activated. Possibly, WRKY70 is a signal integrator from the mutually antagonistic SA and JA pathways (Li *et al.*, 2004).

The *Arabidopsis* *NPR1* gene can enhance disease resistance in rice. Four rice homologues of *Arabidopsis* TGA bZip factors interact with *Arabidopsis* NPR1 in a two-hybrid screen (Chern *et al.*, 2001). A rice *NPR1* homologue was isolated and over-expressed in rice leading to high levels of resistance to bacterial leaf blight (Chern *et al.*, 2005). This strongly suggests that monocot and dicot plants share a conserved signal transduction pathway controlling NPR1-mediated resistance.

### 8.3.4 SA and other regulatory proteins

Other regulators in the SA-signaling pathway were sought using genetic screens to find suppressors of *npr1*. A recessive mutant, *sn1* (suppressor of NPR1 inducible) was found with wildtype basal levels of SA and PRs. The *sn1* mutation fully induces PR expression and pathogen resistance in the *npr1* background upon treatment with SA. In wildtype plants SNI1 may act downstream of NPR1 and NPR1 activity represses SNI. In agreement with this model, SNI1-GFP was found in the nucleus. Although no function was found for SNI, homologues were observed in several other plant species (Li *et al.*, 1999; Durrant & Dong, 2004). A recent study has linked the SA activation of PRs in the *npr1/sn1* double mutant to Whirly transcription factors (Desveaux *et al.*, 2004). Members of this family of transcription factors characteristically bind to single-stranded DNA and function in the chloroplast as well as in the nucleus (Desveaux *et al.*, 2002; 2005). In *Arabidopsis*, AtWhy1-binding activity is induced by SA, but independently of NPR1 (Desveaux *et al.*, 2004). Possibly, as suggested by Desveaux *et al.* (2005), AtWhy1 is involved in the activation of SA-induced PR expression in the *npr1/sn1* double mutant (Fig. 8.2).

### 8.3.5 SA and the mobile signal

Initially, SA was proposed to be the phloem-mobile signal moving from a lower infected leaf to the upper parts where it activates SAR. Grafting and leaf excision experiments indicate that while SA is a necessary component for the induction of SAR, it is not the primary mobile signal exported from the infected leaf (reviewed in Nawrath *et al.*, 2005). While transport of SA could be demonstrated between a lower and an upper leaf, the relative importance of this movement for SAR-induction could never be fully established, as discussed in Nawrath *et al.* (2005).

MeSA, the volatile metabolite of SA, was then proposed to mediate systemic signaling in SAR. MeSA was shown to induce resistance not only in the uninfected parts of the same plant but also in neighbouring plants (Shulaev *et al.*, 1997). This result has gained further support by the recent identification of SABP2, an SA-binding protein with a strong esterase activity on MeSA (Forouhar *et al.*, 2005). Interestingly, the *SAMT* gene, encoding a methyltransferase that catalyses the biosynthesis of MeSA, was isolated and its expression shown to be localized at the site of damage (Ross *et al.*, 1999; Chen *et al.*, 2003). This suggests the following scenario: after a localized infection, MeSA is produced and acts as a long-distance signal that is converted back to SA by SABP2. This model needs to be reconciled with the grafting results of Gaffney *et al.* (1993). In their experiments, SAR takes place when wildtype tobacco scions are grafted onto TMV-inoculated NahG rootstocks. To make the above model plausible, there should still be enough MeSA made from SA in the rootstocks despite the activity of the *NahG* gene. Careful localization studies of the NahG protein in relation to the site of SA synthesis and conversion might perhaps help to clarify this dilemma.

An *Arabidopsis* mutant was found with a dysfunction in DIR1, a putative apoplastic lipid-transfer protein. The *dir1-1* mutant is defective in systemic, but not in local induced resistance, suggesting the involvement of lipid-derived molecules in the transmission of SAR (Maldonado *et al.*, 2002). A lipid-derived molecule as a signal for SAR would be in agreement with the lipase activity of SABP2, as well as with the lack of SAR in the *eds1* and *pad4* mutants, both defective in lipase-like proteins (Falk *et al.*, 1999; Jirage *et al.*, 1999) or the *sfd1* impaired in glycerolipid biosynthesis (Nandi *et al.*, 2004).

A mobile peptide signal was also proposed (Xia *et al.*, 2004). T-DNA-enhancer-trap screening identified the constitutive disease resistance 1 (*CDR1*) gene encoding an apoplastic aspartic protease. Over-expression of *CDR1* causes dwarfing and resistance to virulent *P. syringae*. These phenotypes are linked to SA-dependent activation of micro-oxidative bursts and activation of various defence-related genes. Antisense expression of *CDR1* results in diminished resistance to avirulent *P. syringae* and increased susceptibility to virulent strains. CDR1 accumulates in the intercellular fluid in response to infection and generates a small mobile signal. CDR1 activity is inhibited by the protease inhibitor pepstatin and by mutations in the protease active sites. Thus, it was proposed that CDR1 mediates a peptide signal system involved in the activation of systemically inducible resistance mechanisms. A precedent for a peptide signal exists with the wound-induced systemin (Ryan & Pearce, 2003). It will be interesting to learn more about the structure and mode of action of this novel putative peptide signal.

### 8.3.6 SA and global gene expression

The technical advances in DNA microarray gene expression technology has added to our knowledge on SA-dependent genes. Originally, genes induced by SA and its functional analogues were mostly confined to members of the *PR* gene family (Van Loon & Van Strien, 1999) and PR1 has become a major marker gene for SA and SAR. A microarray prepared with 2375 selected expressed sequence tags (ESTs) with a biased representation of putative defence-associated and regulatory genes was used to measure changes in expression levels in *Arabidopsis* plants after inoculation with an avirulent pathogen or with various signals. Treatment with SA, MeJA and ET resulted in highly increased abundance of 191, 221 and 55 mRNAs, respectively (Schenk *et al.*, 2000). This represented the largest number of SA-induced genes that had been described at that time. A substantial number of genes were regulated by multiple treatments. In particular, SA and MeJA treatments share the largest number of co-induced and co-repressed genes. The results of Schenk *et al.* (2000) confirmed the existence of a network of regulatory interaction and coordination occurring among different plant defence pathways.

Changes in gene expression associated with the induction or maintenance of SAR were then studied using a DNA microarray representing approximately 7000 genes (Maleck *et al.*, 2000). The patterns of gene expression were compared under 14 different SAR-inducing or SAR-repressing conditions. Results showed 413 ESTs



exhibiting a 2.5-fold change or more in at least two SAR-relevant samples. Hierarchical “clustergram” and “self-organizing maps” (SOMs) were used to define groups of co-regulated genes. Interestingly, *PR1* used as a marker for SAR, clustered in a group (Som c3) containing 45 ESTs from a maximum of 31 genes that are likely to function in SAR. These ESTs are all strongly expressed after infection with the incompatible *P. parasitica* as well as after treatment with BTH, the functional analogue of SA. These genes are also activated in uninfected SAR tissue and depend on NIM1/NPR1. Furthermore, the only *cis*-acting regulatory element to appear in all known promoters from the Som c3 gene cluster is the WRKY binding site. NIM1/NPR1 was proposed to mediate a WRKY-dependent de-repression of genes in the PR1 cluster. Alternatively, NIM1/NPR1 might mediate the rapid expression of a subset of WRKY proteins that themselves regulate WRKY-dependent SAR genes (Maleck *et al.*, 2000). Chen *et al.* (2002) confirmed positive and negative interacting SA- and JA/ET- pathways for the expression or repression of transcription factors in *Arabidopsis* upon infection with bacterial pathogens.

Global-expression phenotyping using a form of hierarchical clustering was used on a 8 kb Affymetrix DNA chip (Glazebrook *et al.*, 2003). This approach defined groups of mutations with similar effects on gene expression and groups of similarly regulated genes. For instance, mutations affecting SA signaling were observed to form two groups: one known to affect only SA signaling (comprised of *eds4*, *eds5*, *sid2* and *npr1-3*); and the other affecting SA signaling as well as another unknown process (comprised of *pad2*, *eds3*, *npr1-1*, *pad4* and NahG). Interestingly, important differences were observed between the group of genes expressed in NahG and in the SA biosynthetic mutant *sid2*, demonstrating that NahG has a broader effect on gene expression than simply the elimination of SA. This was confirmed in another study using a genetic approach (Heck *et al.*, 2003). A third group of mutants affected ET and JA signaling and comprised the *eds*, *pad*, *ein2* and *coi1* mutants. The results further document mutual inhibition between SA- and JA-dependent signaling, the requirement for combined pathways, for example JA and ET, and allow the placement of mutants with undefined roles in the signaling network (Glazebrook *et al.*, 2003).

Defence signaling pathways overlap as demonstrated recently for response to pathogens and insects in *Arabidopsis* (De Vos *et al.*, 2005). The kinetics of microbial pathogen- and herbivorous insect-induced signals were determined in relation to global gene expression profiles using Affymetrix ATH1 whole-genome GeneChips. Each combination of assaults elicited specific SA, JA and ET signatures that were translated into a complex gene expression pattern where stress-related genes are over-represented. An overlap exists between genes expressed in response to pathogens or insect with widely different modes of attack. For example, among the consistent changes induced by *A. brassicicola*, *Pieris rapae* and *F. occidentalis*, more than 50% are also induced consistently by *P. syringae*. Curiously, although JA biosynthesis is stimulated after each of these attackers, the overall JA-responsive gene expression pattern is specific to each attacker. Another surprising observation concerns the case of aphid feeding. These insects caused the least symptoms of all attackers tested, but induced the largest number of consistent changes in gene



expression without detectable changes in SA, JA and ET levels. Many pathogen or insect specific sets of genes have also been observed in comparable studies (Reymond *et al.*, 2000; Glazebrook *et al.*, 2003; Tao *et al.*, 2003; van Wees *et al.*, 2003; Verhagen *et al.*, 2004). Thus, while SA, JA and ET signals orchestrate an important proportion of the defence response, other regulatory mechanisms, such as cross-talks between pathways or additional hitherto unidentified signals, combine to fine-tune the response of the plant to each interaction.

### 8.3.7 SA and virus resistance

In tobacco and *Arabidopsis*, SA inhibits the replication, cell-to-cell and long-distance movement of several RNA viruses (reviewed in Singh *et al.*, 2004). This multi-layered defence system was proposed to offer an effective viral defence: if a virus escapes one layer, it might be affected by others (Singh *et al.*, 2004). The SA-dependent viral defence operates against RNA as well as DNA viruses. While SA-dependent PR expression is dependent on NPR1, the SA-dependent viral defence appears independent of NPR1, but relies on the ROS generated in the mitochondrion. Alternative oxidase (AOX) was suggested to play an important role in virus resistance based on pharmacological studies (reviewed in Singh *et al.*, 2004) as well as on studies with plants expressing high levels of AOX (Murphy *et al.*, 2004). The inhibition of the mitochondrial electron transport chain by SA leads to an increase in ROS, the amplitude and timing of which are under the negative control of an AOX.

These mitochondrial signals might be perceived by an as-yet unidentified sensor, probably *via* thiol/disulphide exchange (Dutilleul *et al.*, 2003) and activate the expression of genes involved in viral defence (Gilliland *et al.*, 2003; Singh *et al.*, 2004). Interestingly, in tobacco and *Arabidopsis*, SA also induces the AOX-independent expression of an RNA-dependent RNA polymerase gene that is involved in RNAi-mediated antiviral defence (Xie *et al.*, 2001; Yu *et al.*, 2003). This might constitute an additional branch in the SA-signaling pathway for viral defence (Fig. 8.2).

## 8.4 Conclusions

Research on SA took a definitive turn in the 1990s with the first publications on the possible role of SA in the regulation of induced disease mechanisms and the initiation of molecular approaches to studies on SAR. This review reflects the bias of its authors for this particular area and has somewhat neglected other roles this hormone can play in the life of plants. Future research will enable a better description of the involvement of SA in the integration of signals associated with processes such as flowering, thermotolerance or cold stress.

There are still many bottlenecks in our understanding of SA signaling. In the near future, we can expect to learn more about the regulation of its biosynthesis, and of its concentration at sites of infection and at remote sites. The mode of action of SA, its putative binding site(s), the resulting responses as well as the integration of the

SA response with other signals are further areas where exciting findings are expected. Undoubtedly, this fundamental knowledge will have an impact in agriculture, with the development of resistant varieties or plant immunization strategies.

Our advances have been fostered by the sophisticated methodology that has become increasingly available, such as genome-wide expression analysis, proteomics, metabolomics, forward and reverse genetics, bioinformatics and biomathematics. More experimental data can now be obtained technically than there is time to analyse, emphasizing the importance of the originality and creativity of the individual scientist in the design of critical experiments.

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## 9 Hormone distribution and transport

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### 9.1 Concepts and definitions

A number of bioactive substances found in plants exhibit some similarities to hormones in the endocrine system of animals. However, plants have developed an amazing variety of mechanisms regulating phytohormone distribution and activity. Distribution of phytohormones can depend on a concentration gradient established in the plant body, or it can be mediated by free diffusion, as is the case for the gaseous compound ethylene (Davies, 2004). On the other hand, polar transport of the phytohormone auxin, which occurs in a strictly unidirectional fashion, depends on a complex machinery of carrier proteins, mediating cellular uptake and efflux steps that ensure establishment and maintenance of hormone gradients in the developing plant body. In this chapter we address and compare phytohormone distribution of three examples, representing the quite distinct mechanisms in the control of phytohormone activities: auxin, which is actively transported throughout the entire plant body; gibberellin (GA), which appears to be transported in some circumstances and brassinosteroid (BR), for which no clear-cut evidence for transport is so far available. Moreover, we address points concerning regulatory cross-talk between these growth regulators, integrating distinct mechanisms of hormone distribution in the control of plant growth and development.

### 9.2 Auxins: distribution and transport

#### 9.2.1 *Auxin distribution: old views and new developments*

Auxin has a prominent position among the classical plant hormones since it mediates multiple aspects of plant growth and development. Besides its involvement in division, enlargement and differentiation of individual plant cells, auxins also function as signals between cells, tissues and organs (Davies, 2004). A key role in these responses has been attributed to temporal and spatial control of auxin levels (homeostasis), which would be required to initiate and perpetuate developmental events or growth responses that occur because of variation in intrinsic or environmental cues.

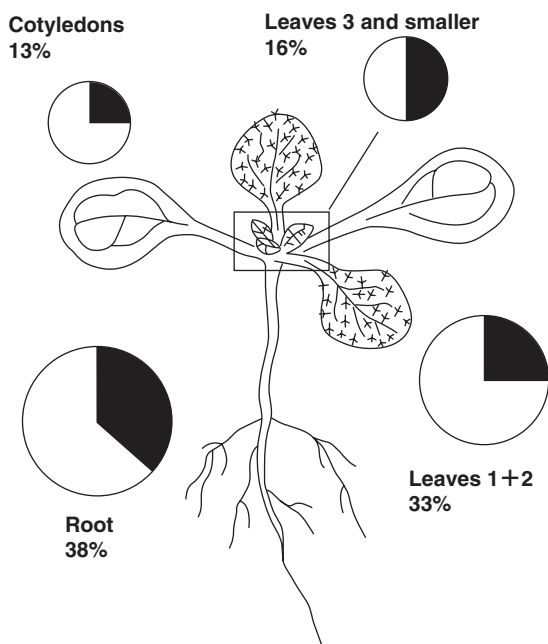
The conceptual framework regarding the biological function of auxin in higher plants is essentially based on the view that auxin is primarily synthesised in the apical parts of the shoot and subsequently delivered to other parts of the plant body where it exerts its activity in the regulation of a variety of growth responses. This

view remained more or less unquestioned for many years, as reflected in an often oversimplified presentation of sites and mechanisms of auxin biosynthesis and distribution. This somewhat unsatisfactory scenario was mainly based on the fact that quantitative analysis of free auxin or inactive auxin conjugates in a given cell file represented a major challenge for analytical phytochemistry. That situation has changed dramatically in the last few years, providing us with a contemporary picture of sites of auxin biosynthesis and auxin distribution during plant development, specifically in the model organism *Arabidopsis thaliana* (Casimiro *et al.*, 2001; Ljung *et al.*, 2001; 2005; Bhalerao *et al.*, 2002).

### 9.2.2 Auxin biosynthesis: not restricted to the shoot anymore

Control of auxin steady-state levels is thought to involve changes in biosynthesis, in metabolism, or in transport into and out of the tissue concerned. The most abundant naturally occurring auxin is indole-3-acetic acid (IAA); but other auxins including indole-3-butyric acid (IBA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) have also been identified in plants (Engvild, 1975; Schneider *et al.*, 1985; Ludwig Müller & Epstein, 1991). Besides these physiologically active auxin forms, inactive auxin conjugates linked to sugars, amino acids or to small peptides have been isolated (Cohen & Bandurski, 1978; 1982; Tam *et al.*, 2000). Importantly, regulation of the ratio between conjugated and de-conjugated forms of auxin could represent an effective means to control steady-state levels of active auxin in a given tissue (Woodward & Bartel, 2005). The oxidative degradation of auxin also has been implicated in the control of auxin concentrations (Hinman & Lang, 1965; Semblner *et al.*, 1981). All these findings are suggestive of a scenario in which a complex interplay between metabolic events mediating auxin biosynthesis, conjugation and degradation would play an important role in cell- and tissue-specific control of auxin homeostasis (Woodward & Bartel, 2005).

With the availability of novel techniques such as micro-scale gas chromatography-coupled mass spectrometry (Edlund *et al.*, 1995), together with skilled hands capable of dissecting plant tissue nearly to the cellular level, it has become possible to study distribution and biosynthesis of auxin at a very high resolution. Analyses performed in *Arabidopsis* confirmed some of the older views, as auxin concentrations were found to be highest in the youngest leaves formed by the shoot apical meristem (SAM) (Ljung *et al.*, 2001). While it turned out that essentially all vegetative organs are capable of synthesising IAA *de novo* (Ljung *et al.*, 2001), in general, rates of auxin biosynthesis were found to be highest in meristematic tissue (Ljung *et al.*, 2001; 2005), also correlating with the important role of auxin in the control of cell proliferation (Willemsen & Scheres, 2004). For example, a comparison of IAA pool sizes in different organs demonstrated that *de novo* auxin synthesis in 10-day-old roots contributes a significant amount to the entire auxin pool synthesised in *Arabidopsis* (Fig. 9.1). Thus, roots apparently do represent a major auxin source. Nevertheless, as demonstrated by labelling and inhibitor studies, a significant amount of auxin found in root tissue derives from shoot sources



**Figure 9.1** Auxin pool sizes in 10-day-old *Arabidopsis* plants, grown under long-day conditions. Circles represent the relative amounts of IAA found in different organs. Black segments within the circles correspond to auxin-synthesised *de novo* within the previous 24 h (reprinted with permission from Ljung *et al.*, 2001).

(Bhalerao *et al.*, 2002; Ljung *et al.*, 2005), raising questions about the biological relevance of two distinct auxin pools found in roots. An answer to these questions came from the analysis of auxin biosynthesis and distribution in a spatiotemporal context (Ljung *et al.*, 2001; 2005; Bhalerao *et al.*, 2002). Auxin found in the roots of very young *Arabidopsis* plantlets is most likely derived from shoot sources, a view supported by the analysis of lateral root emergence on decapitated seedlings. Removal of shoot-derived organs, such as the first true leaves, inhibited lateral root emergence, indicative of a requirement for shoot-derived auxin in the control of lateral root growth at early developmental stages (Bhalerao *et al.*, 2002). By contrast, upon removal of aerial tissues at later developmental stages, lateral root emergence was significantly less affected, indicative of a correlation between root development and an increasing competence of the root to synthesise auxin (Bhalerao *et al.*, 2002). Further support for this idea came from the quantitative analysis of auxin biosynthesis in roots (Ljung *et al.*, 2005). In particular, primary root meristems as well as emerged lateral roots appear to represent important sources for root-synthesised auxin (Ljung *et al.*, 2005). From that it appears that auxin, derived from the root as well as from the shoot, exerts a combinatorial effect on the regulation of auxin-mediated responses in the control of root growth.



### 9.3 Auxin transport

Another means of controlling auxin homeostasis at specific locations involves the controlled, coordinated transport of auxin throughout the plant body. The first indication for a biological relevance of auxin transport dates back to Darwin's experiments on phototropism, which indicated the existence of transported signals (Darwin & Darwin, 1881). Several decades later, Went and Cholodny performed growth experiments suggestive of a scenario in which transport of an externally applied regulator would regulate plant tropisms (Cholodny, 1928; Went & Thimann, 1937) and the responsible substance was subsequently termed auxin by Kögl and Haagen-Smit (1931).

Two physiologically distinct and spatially separated mechanisms appear to be involved in auxin transport over long distances in plants. One mechanism appears to translocate auxin rapidly by mass flow in the mature phloem, whereas another mechanism controls auxin transport in a strictly polar fashion (Morris & Thomas, 1978; Cambridge & Morris, 1996). The major route of such polar auxin transport (PAT) involves relocation of the growth regulator from immature tissues close to the shoot apex downwards towards the root tips. In addition, auxin reaching the root tip appears to be redistributed back towards the base of the root, presumably via the outermost cell layers of the root (Rashotte *et al.*, 2000). Polar transport of auxin is much slower (*ca.* 7–15 mm/h) than mass-flow-dependent auxin distribution, and involves, carrier-dependent, cell-to-cell polar transport (Goldsmith, 1977). In that respect, auxin is unique among plant hormones, as it is actively transported from source organs in young, apical regions to target cells where it might exert its biological activity (Davies, 2004).

#### 9.3.1 Mass-flow-dependent distribution of auxin

There is considerable evidence that IAA is a constituent of phloem sap (Baker, 2000). After entering the phloem in the leaves, IAA would then be passively translocated to and unloaded at auxin sinks. The direction and velocity of auxin translocation in phloem cells thus primarily depends on spatial variations in auxin concentrations (Morris, 1996). However, while substantial quantities of auxin can be found in the phloem sap the role of this auxin pool in auxin-mediated growth responses is still poorly understood (Baker, 2000).

#### 9.3.2 Polar auxin transport

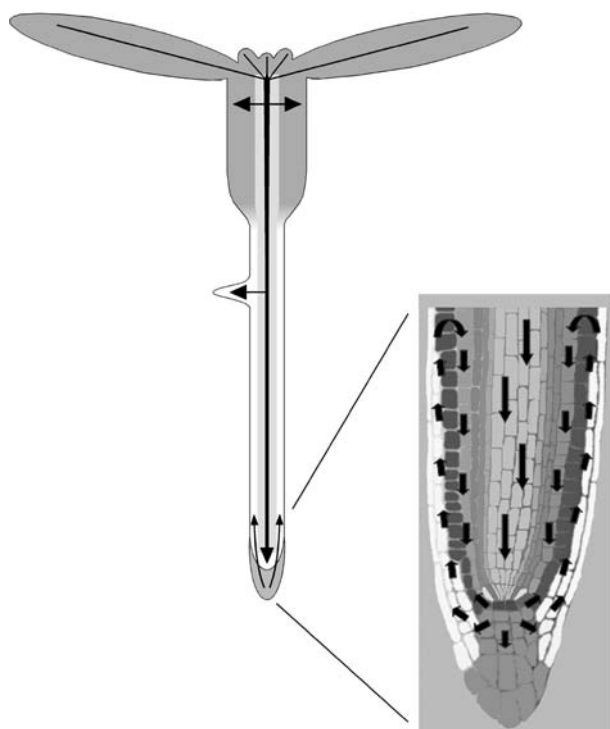
##### 9.3.2.1 Physiological aspects

Unlike mass-flow-dependent distribution, PAT has been shown to exhibit characteristics of a carrier-mediated process that is energy dependent, saturable, and which involves cellular uptake as well as efflux of the growth regulator (Goldsmith, 1977). Long before the first molecular players involved in PAT were characterised,

a number of elegant physiological experiments already predicted the involvement of specific carrier proteins in this process. Specifically, saturable auxin uptake and efflux activities have been demonstrated in single plant cells as well as in tissue segments (Hertel & Leopold, 1963; Rubery & Sheldrake, 1974; Goldsmith, 1977; Davies & Rubery, 1978). Remarkably, these carrier activities exhibit quite distinct substrate specificities. Auxin uptake could be demonstrated for IAA and 2,4-dichlorophenoxyacetic acid (2,4-D), whereas carrier-dependent auxin efflux is efficient for IAA and naphthalene-1-acetic acid (NAA) (Delbarre *et al.*, 1996). Besides differences in substrate specificity, auxin uptake and efflux activities can be distinguished using a set of synthetic auxin transport inhibitors. Auxin efflux inhibitors were characterised in the 1970s and were subsequently shown to inhibit auxin efflux in single cells as well as in entire plants (Katekar & Geissler, 1977; Rubery, 1990). Well-known inhibitors of auxin efflux are 2,3,5-triiodobenzoic acid (TIBA) and *N*-1-naphthylphthalamic acid (NPA), which are both commonly used to study physiological and developmental consequences of inhibition of PAT (Fujita & Syono, 1996; Guerrero *et al.*, 1999). Recently, however, treatment with high concentrations of synthetic auxin efflux inhibitors has been shown to exhibit a quite general effect on the control of membrane protein cycling (Geldner *et al.*, 2001), raising questions about the specificity of these compounds. Interestingly, a group of plant metabolites, namely aglyconic flavonoids, appear to inhibit auxin efflux, leading to the suggestion that these compounds might act as natural regulators of PAT (Jacobs & Rubery, 1988; Brown *et al.*, 2001). However, these compounds also appear to interfere with a large variety of biological processes (Taylor & Grotewold, 2005). Thus, a specific role of flavonoids in the regulation of PAT remains to be determined. In addition, substances interfering with carrier-mediated cellular uptake of auxin have been identified (Imhoff *et al.*, 2000). Specifically, 1-naphthoxyacetic acid (1-NOA) and 3-chloro-4-hydroxyphenylacetic acid (CHPAA) were shown to inhibit auxin uptake in tobacco culture cells (Imhoff *et al.*, 2000).

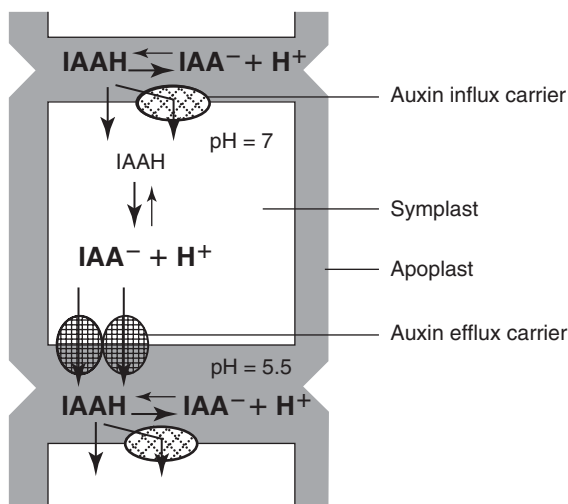
In addition to the pharmacological characterisation of PAT, the sites and tissue that mediate such transport have been well characterised. Auxin is basipetally transported from the apex to the base of the shoot (Lomax *et al.*, 1995; Fig. 9.2). PAT in the stem has been localised to the vascular cambium (Morris & Thomas, 1978). In addition to basipetal auxin transport, evidence for lateral auxin transport has been found in the shoot as well (Morris & Thomas, 1978; Epel *et al.*, 1992). Once auxin has passed the shoot-to-root-junction, further polar transport of auxin proceeds in the root vasculature towards the root tip. From the root tip, auxin is then redistributed upwards into the root elongation zone (Rashotte *et al.*, 2000; Fig. 9.2). Thus, roots exhibit both acropetally and basipetally oriented auxin streams, both of which have been implicated in a variety of auxin-mediated growth responses, including root elongation, lateral root formation and root tropisms (Rashotte *et al.*, 2000; Casimiro *et al.*, 2001; Fu & Harberd, 2003).

A conceptual framework for the mechanisms underlying the coordinated polar distribution of auxin was proposed in the mid-1970s. The chemiosmotic hypothesis



**Figure 9.2** Sites and direction of PAT in higher plants (arrows). Auxin from the shoot is transported towards the root tip. Auxin transport towards the root tip coincides with lateral distribution of the growth regulator, which appears crucial for the control of cell elongation and for the formation of lateral roots. Transport from the root tip is presumed to regulate meristem size and root gravitropism via differential auxin distribution in the root elongation zone (reprinted from Benjamins *et al.*, 2005).

for PAT (Fig. 9.3) was proposed by Rubery & Sheldrake (1974) and Raven (1975), and is based on the observation that only the protonated form of auxin (IAAH) can efficiently move across the plasma membrane, whereas the auxin anion ( $\text{IAA}^-$ ) is unable to cross the membrane. In the relatively acidic environment of the cell wall, a fraction of IAA is in its protonated form (IAAH), which could therefore efficiently enter the cell via concentration gradient-dependent diffusion. Moreover, activity of auxin uptake carriers could further reinforce cellular uptake of auxin. The higher pH found in the cytoplasm then forces the majority of IAAH to dissociate. As a result, the highly polar  $\text{IAA}^-$  anion is “trapped” in the cell because of its poor membrane permeability. Further transport of  $\text{IAA}^-$  thus requires specific carriers that mediate the efflux of auxin into the surrounding apoplast. The polarity of auxin transport would be the result of an asymmetric distribution of efflux carriers in cell membranes that allows auxin transport to proceed in only one direction. Polar auxin export from a single cell would then multiply in a file of neighbouring cells that all exhibit the same asymmetric distribution of auxin efflux carrier proteins.



**Figure 9.3** *Chemiosmotic model for PAT.* In the acidic apoplast, a fraction of IAA (about 15%, based on a pH of 5.5 and pK<sub>a</sub> of 4.7) is present in the protonated form (IAAH) and can enter the cell by diffusion or via active import by auxin influx carriers. Due to a higher pH inside the cell, IAA is deprotonated and thereby trapped in the cell. Active efflux mediated by presumptive auxin efflux carriers is required for further transport of auxin. Asymmetric distribution of the efflux carriers at the plasma membrane would ensure directional auxin transport (modified from Lomax *et al.*, 1995).

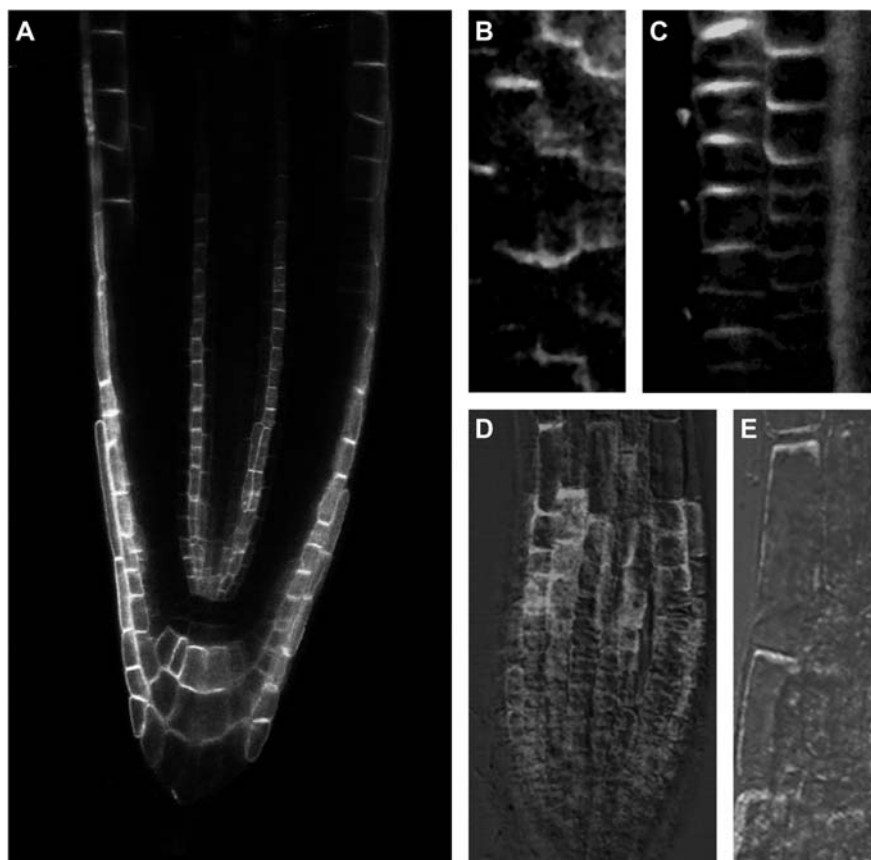
This classical hypothesis has remained essentially unchallenged for the last 30 years, and was recently supported by the identification of candidate proteins, seemingly involved in facilitating uptake and efflux of auxin in a concerted mechanism that controls the polar distribution of auxin (Morris *et al.*, 2004; Blakeslee *et al.*, 2005; Paponov *et al.*, 2005).

### 9.3.2.2 Auxin transporters

In contrast to mass-flow-dependent auxin transport, PAT can be strictly regulated by the plant via control of influx and efflux carriers. Most of the research to date has been on *Arabidopsis thaliana*, in which the following set of putative auxin carriers have so far been identified:

- (1) The AUX1/LAX influx carriers comprising four members of amino acid permease-type proteins (Parry *et al.*, 2001).
- (2) The PIN efflux carriers comprising eight members resembling putative bacterial transporters (Blakeslee *et al.*, 2005; Paponov *et al.*, 2005).
- (3) The MDR/PGP efflux carriers comprising many members homologous to multidrug resistance/P-glycoproteins transporters (Martinoia *et al.*, 2002; Blakeslee *et al.*, 2005).

A role for these proteins in PAT is primarily demonstrated by the phenotypes of the corresponding mutants, such as altered PAT and associated growth defects. Their role as actual carriers has been implied from two features: firstly, their highly



**Figure 9.4** *Expression and subcellular localisation of putative auxin carriers.* (a) Expression of AUYFP in an *Arabidopsis* root meristem. Expression is most abundant in lateral root cap cells. Note the polar subcellular polarisation of the reporter protein. Immunolocalisation of (b) PIN1 and (c) PIN2 in *Arabidopsis* root meristems. PIN1 exhibits a highly polar distribution in the stele, whereas PIN2 is predominantly found in lateral root cap, epidermis and cortical cells. (d) Immunolocalisation of PGP1 in *Arabidopsis*. The protein exhibits a less polar distribution in the cell division zone of the root meristem. (e) Immunolocalisation of PGP4 in *Arabidopsis* root epidermis cells demonstrates a polar distribution of the protein. Images were kindly provided by Malcolm Bennett and Ranjan Swarup (a) and by Angus Murphy and Wendy Peers (d and e).

polar localisation in cells (Fig. 9.4) which supports the chemiosmotic hypothesis, and secondly, their homology to other permeases/transporters.

To date, the PINs have featured most prominently in research on PAT. Their role in PAT is further supported by the strong phenotypes of the *pin1* (pin-formed inflorescences) and *pin2* mutants (agravitropic), which clearly demonstrate PAT deficiencies (Chen *et al.*, 1998; Gälweiler *et al.*, 1998; Luschnig *et al.*, 1998; Müller *et al.*, 1998). However, whether or not the PINs are true auxin carriers is less certain – although

PINs are known to be homologous to bacterial proteins with mostly predicted functions in transport across membranes. This similarity, however, is restricted to the transmembrane region, whereas the large hydrophilic loop can only be found in plant PIN proteins (Chen *et al.*, 1998; Luschnig *et al.*, 1998).

The MDR/PGPs are implicated in PAT as they can bind the auxin efflux inhibitor NPA, and due to the PAT-related defects exhibited by *pgp1* and *pgp19/mdr1* mutants (Noh *et al.*, 2001; Murphy *et al.*, 2002; Geisler *et al.*, 2003; 2005). Moreover, although MDR/PGPs are less polarly localised (Fig. 9.4; Geisler *et al.*, 2005), their homology to known ABC transporters is highly suggestive for a function as auxin efflux transporters.

As the chemiosmotic model predicts that auxin influx can be passive, the requirement for a specific influx carrier such as AUX1 seems at first redundant. However, the agravitropic phenotype of *aux1*, the polar localisation of AUX1 and its homology to known permease-type proteins supports its role as an influx carrier. In addition, *aux1* is more resistant (in root elongation assays) than wild-type (WT) plants to auxins-like 2,4-D and IAA, suggesting that active auxin uptake is crucial for at least some PAT-related processes, particularly in the root tip (Bennett *et al.*, 1996; Marchant *et al.*, 1999; Swarup *et al.*, 2001).

To be considered true auxin carriers, it is essential to prove actual auxin transport activity for these putative carriers. This has been a difficult task for several reasons. Firstly, any measurement of “activity” refers to the amount of auxin transported or retained in whole cells or tissues. Thus, what is being measured is not the activity of a specific carrier but the combined effect of *all* influx and efflux activities, including passive diffusion. Unfortunately, comparisons between WT and mutants of the various carriers do not overcome this problem due to the redundancy of the carriers, for example the absence of one PIN can be compensated by an increase in the other PINs (Blilou *et al.*, 2005; Paponov *et al.*, 2005; Vieten *et al.*, 2005). Secondly, evidence of auxin transport activity can also mean that the “carriers” are required as part of a complex that transports auxin. This might be particularly true for the PINs, as they are not homologous to established transporters/permeases and their function in PAT is thus less certain. Thirdly, one has to show specificity for auxin. This is probably most relevant for the MDR/PGPs, as MDRs can have a broad range of substrates. Measurements of auxin transport performed in heterologous systems (i.e. expressing the plant carriers in non-plant hosts) are also subject to these limitations.

With the above limitations in mind, there is evidence to support auxin transport activity for AUX1 and PIN2 in root (Chen *et al.*, 1998; Marchant *et al.*, 1999), and some weak evidence when PIN2 is expressed in yeast (Chen *et al.*, 1998). PGP1 appears to facilitate auxin transport when expressed in yeast and HeLa cells (Geisler *et al.*, 2005), but Noh *et al.* (2001) failed to find auxin transport activity in the same yeast system for PGP19.

### 9.3.2.3 Regulation of the carriers

As with any cellular process, regulation of PAT can be directed at changing either the *amount* of protein (e.g. by increasing transcription or reducing degradation of protein)

or the actual *activity* of the protein (e.g. by covalent modification or allosteric activation). However, it is often not possible to distinguish between these two specific changes. In most cases, PAT is simply measured in tissue segments, or more often, a secondary effect of PAT is observed (e.g. root length or gravitropic response).

*PIN* mRNA expression is tissue- and developmental-stage-specific (Paponov *et al.*, 2005; Vieten *et al.*, 2005) and in some cases circadian-regulated (Harmer *et al.*, 2000). Specific changes in mRNA (or expression of transcriptional reporter genes) have been reported for the PINs and MDR/PGPs following a variety of treatments that can be linked to PAT. These include treatment with IAA and auxin transport inhibitors NPA and TIBA (Noh *et al.*, 2001; Blakeslee *et al.*, 2004; Peer *et al.*, 2004; Geisler *et al.*, 2005), mechanical or gravitational stimulation (Kimbrough *et al.*, 2004) or dark/light transition (Noh *et al.*, 2001; Geisler *et al.*, 2005). However, changes in mRNA are indicative of transcriptional control, but may not be matched by corresponding increases in protein (e.g. 200-fold increases in *PIN* mRNA levels were matched by little or no increase in *PIN* protein; Peer *et al.*, 2004). This illustrates the importance of following up any mRNA analysis with protein analysis.

Levels of mRNA are affected in certain mutant backgrounds and such data may be useful in identifying possible components in the control of PAT. For example, *PIN3* and *PIN4* are downregulated in *ifl1* (Zhong & Ye, 2001). As *ifl1-2* mutants have reduced PAT and exhibit the distinct *pin1* phenotype, that is *pin*-formed inflorescence stems, this indicates that IFL (a homoeodomain-leucine zipper protein) could be required for *PIN* control.

There is a scarcity of data showing actual quantitative changes (e.g. Western blot analysis) in total carrier protein levels as part of a regulatory process. To an extent this certainly reflects the difficulties of dealing with plant membrane proteins, but it is also due to a unique aspect of the carriers, that is their function is dependent on their membrane localisation. Thus, adjusting the amount of carrier protein at the membrane compared to the amount that is in internal compartments could affect their activity. Changes in the rate of cycling or the polarity of distribution also could affect the rate or direction of PAT. It has also been suggested that the cycling of PINs may be part of their transport activity, such that auxin is transported in cargo vesicles which are then exocytosed in the manner of mammalian neurotransmitter release (Morris *et al.*, 2004). Studying the distribution of carrier proteins between the membrane and internal compartments (or changes in polarity) requires *in situ* analysis (e.g. immunolocalisations or fluorescent reporter proteins). The majority of reported results comprise such visual analysis and have revealed fascinating insights as to how carrier proteins are regulated. However, the disadvantage is that such analysis is more subjective, particularly for quantitative comparisons.

Membrane localisation of the carriers can be affected by the following:

- (1) Gravitropic and phototropic stimulation, which leads to relocation of *PIN* proteins (Friml *et al.*, 2002; Blakeslee *et al.*, 2004). Moreover, control of *PIN2* endocytosis (via a putative auxin-dependent mechanism) has also been implicated in the root gravitropic response (Paciorek *et al.*, 2005).



These processes may mediate asymmetric auxin redistribution and cell elongation during tropic responses.

- (2) Auxin treatment, which can increase the internalisation of PIN1 in hypocotyl (Blakeslee *et al.*, 2004) and root tip cells (Peer *et al.*, 2004). This is also supported by analysis of the *tt4* mutant, roots of which have higher auxin levels than WT and a diffuse PIN1 pattern (Peer *et al.*, 2004). In contrast to these data, auxins may also enhance PIN1 or PIN2 membrane localisation (Peer *et al.*, 2004; Paciorek *et al.*, 2005).
- (3) The vesicular cycling inhibitor brefeldin A (BFA), which has been widely used to demonstrate the cellular internalisation and cycling of PIN1 in roots (Steinmann *et al.*, 1999; Geldner *et al.*, 2001; 2003; Paciorek *et al.*, 2005). PIN3 can also be internalised by BFA (Friml *et al.*, 2002), while the data on PIN2 are more equivocal (Geldner *et al.*, 2003; Paciorek *et al.*, 2005; Shin *et al.*, 2005). The BFA-sensitive cycling of PIN1 requires GNOM, a GDP/GTP exchange factor for small G-proteins (Geldner *et al.*, 2003) and in the *gnom* mutant embryo, PIN1 polar distribution is disorganised (Steinmann *et al.*, 1999). BFA has also been shown to internalise AUX1, although this required much higher BFA concentrations (100  $\mu$ M) than that required to see an effect on AUX1-dependent phenotypes (1  $\mu$ M; Grebe *et al.*, 2002) and is in contrast to studies that show a lack of effect of BFA on auxin influx (Delbarre *et al.*, 1998; Morris & Robinson, 1998).
- (4) The auxin transport inhibitors TIBA and NPA, which can prevent the reversibility of the BFA-induced internalisation of PINs (Geldner *et al.*, 2001). Also, in *big/tir3/doc1* mutants, NPA by itself can cause internalisation of PIN1 (Gil *et al.*, 2001). However, doubts have been raised (Petrásek *et al.*, 2003) about the high amounts of NPA required to see these effects (up to 200  $\mu$ M NPA, whereas auxin efflux is already inhibited by 1–10  $\mu$ M NPA). Notwithstanding such doubts, the observation in *big/tir3/doc1* is interesting, as BIG has been implicated in mediating the effect of auxin on PIN cycling (Paciorek *et al.*, 2005).
- (5) Various mutations; for example in the PINOID kinase mutant *pid* or in PID over-expression plants, the normal apical or basal locations of PIN1, PIN2 and PIN4 are shifted (Friml *et al.*, 2004). A diffuse rather than polar patterning of PIN1 is seen in flavonoid-deficient mutants *tt4* (Peer *et al.*, 2004) and in *mdr1pgp* hypocotyls<sup>1</sup> (Noh *et al.*, 2003); the latter raises interesting possibilities of links between the two carrier families. PIN1 polarity and PIN3 distribution are altered in the sterol methyltransferase mutant *smt1/orc* (Willemsen *et al.*, 2003). In all of these mutants, PAT and PAT-dependent processes are also affected, thus illustrating the correlation between PAT and correct PIN positioning. However, whether or not PAT is dependent on PIN localisation, or *vice versa*, is still an open question.

<sup>1</sup>This effect in *mdr1pgp* has been reported to be dependent on Triton-X100 treatment of the samples (Blakeslee *et al.*, 2005).

As mentioned above, the activity of the carriers is closely tied to their membrane localisation. Conditions that perturb localisation of the PINs have generally been shown to affect PAT rates. For example, BFA treatment causes intracellular accumulation of PINs, correlating with an inhibition of auxin efflux or PAT in tobacco suspension culture cells (Delbarre *et al.*, 1998; Petrášek *et al.*, 2003; Paciorek *et al.*, 2005), zucchini hypocotyls (Morris & Robinson, 1998) and *Arabidopsis* (Geldner *et al.*, 2003). In agreement with its effects on auxin transport, BFA also affects processes dependent on PAT, for example it inhibits lateral root growth, decreases root and hypocotyl length, and inhibits gravitropism (Geldner *et al.*, 2001; 2003). BFA also affects the polarity of root hair initiation, a process that is auxin gradient dependent and has been linked to AUX1 activity (Grebe *et al.*, 2002).

In addition to the effects of cellular mislocalisation of the carriers, the following have also been shown to affect PAT, although the mechanisms or the connection to the auxin carriers are uncertain:

- (1) Various protein kinase and phosphatase inhibitors in general inhibit auxin efflux or basipetal PAT (Bernasconi, 1996; Delbarre *et al.*, 1998; Shin *et al.*, 2005); but may also enhance basipetal PAT (Rashotte *et al.*, 2001; Shin *et al.*, 2005). The latter is in agreement with the increased PAT seen in the *rcn* mutant, which has reduced phosphatase 2A activity (*RCN* codes for a regulatory sub-unit of protein phosphatase 2A; Garbers *et al.*, 1996; Deruère *et al.*, 1999; Rashotte *et al.*, 2001). Moreover, PINOID kinase also affects auxin distribution, that is a decrease in IAA accumulation in root tips (Friml *et al.*, 2004).
- (2) Acidification of the intracellular pH of cells reduces active auxin influx and efflux, while alkalinisation of the extracellular pH reduces influx (Delbarre *et al.*, 1998). The transient alkalinisation observed in root columella cells following gravistimulation has been suggested to be involved in the control of PIN activity or localisation (Boonsirichai *et al.*, 2003).
- (3) NPA is widely used as an inhibitor of general auxin efflux and PAT (e.g. Bernasconi, 1996; Murphy *et al.*, 2000; Brown *et al.*, 2001; Gil *et al.*, 2001; Petrášek *et al.*, 2003). The mechanism of action is not entirely clear but there is accumulating data to make some reasonable hypotheses. NPA has been shown to bind directly to distinct proteins including the MDR/PGP carriers (Noh *et al.*, 2001; Murphy *et al.*, 2002). Moreover, MDR/PGP interacts with the cyclophilin-like protein TWISTED DWARF1 (TWD1); this interaction is disrupted by NPA, and the *twd1* mutant has decreased PAT (Geisler *et al.*, 2003). These data suggest that the inhibitive effect of NPA on auxin efflux could be due to the direct binding of NPA to MDR/PGP carriers. Alternatively, as PIN1 is mislocalised in *mdr/pgp* mutants, a scenario where the PINs and the MDR/PGPs operate in a complex has been postulated (Noh *et al.*, 2003; Blakeslee *et al.*, 2005). Apart from MDR/PGP proteins, NPA also binds to aminopeptidases (Murphy *et al.*, 2002) and a glutathione-S-transferase (GSTF2; Zettl *et al.*, 1994; Murphy *et al.*, 2002; Smith *et al.*, 2003). Intriguingly, GSTF2 also binds auxin and has been suggested as a cytoplasmic escort for auxin (Smith *et al.*, 2003).

- (4) Flavonoids have a negative effect on PAT (Brown *et al.*, 2001) and can inhibit auxin efflux/leakage from roots (Murphy *et al.*, 2000) and hypocotyls (Jacobs & Rubery, 1988). In agreement, defects in flavonoid biosynthesis in the *tt4* mutant correlate with elevated PAT and with phenotypes associated with elevated PAT (Murphy *et al.*, 2000; Buer & Muday, 2004; Peer *et al.*, 2004).

The structural resemblance of flavonoids to NPA, and their ability to compete for NPA-binding sites on the various NPA-binding proteins, indicates that NPA may inhibit PAT by mimicking the natural flavonoids (Jacobs & Rubery, 1988; Bernasconi, 1996; Murphy *et al.*, 2000; Smith *et al.*, 2003; Taylor & Grotewold, 2005). In addition, NPA can compensate for flavonoid deficiencies in *tt4* mutants (Murphy *et al.*, 2000; Peer *et al.*, 2004). An intriguing possible mechanism of flavonoid/NPA action arises from the fact that flavonoids are known to compete for ATP-binding sites in ATP-binding proteins and in this way act as physiological negative regulators (Conseil *et al.*, 1998; Taylor & Grotewold, 2005). In particular, flavonoids can displace ATP in mouse MDR/PGP homologues (Conseil *et al.*, 1998). Thus, by analogy with the situation in mammals, NPA/flavonoid-type molecules might bind to the nucleotide-binding region of plant MDR/PGPs and in this way inhibit MDR/PGP activity in catalysing auxin transport.

### 9.3.3 Conclusion: a joint effort required for auxin transport?

We mentioned earlier that measurements of PAT reflect the sum of all influx and efflux paths of auxin in cells or tissues, and that it was thus not possible to measure activities of the individual carriers. However, the fact that PIN localisation can change has given researchers a good tool to observe direct effects on these carriers in response to a certain treatment. Following up this with PAT measurements (or observing secondary effects of PAT) allows further postulations on the putative role of the PINs in mediating PAT.

The technical inability to assign any observed changes in PAT to specific carriers is a disadvantage when analysing the role of each carrier, but it also simply reflects the true situation, that is the carriers act together. For example, an emerging model of PAT is that the PINs and the MDR/PGPs form a complex to facilitate auxin transport against an electrochemical gradient (Blakeslee *et al.*, 2005). The AUX1 influx carrier would enhance the direction of PAT in tissues where tight regulation of this process is absolutely crucial for a response (e.g. gravitropic response in the root tip). And no doubt future research will also discover other proteins involved in the carrier machinery.

## 9.4 GAs: distribution and transport

The second major hormone group to be discussed here are the GAs. The GAs are potent promoters of elongation growth, in both shoots and roots. Their physiological characterisation has long been associated with dwarf mutants, many of which are

grossly deficient in bioactive GAs. When applied to such mutants, GAs dramatically promote growth, even in parts of the plant distant from the application site. However, this does not mean that *endogenous* GAs are normally transported long distances within the WT plant, as the ensuing discussion will demonstrate.

#### 9.4.1 *Seeds and fruits*

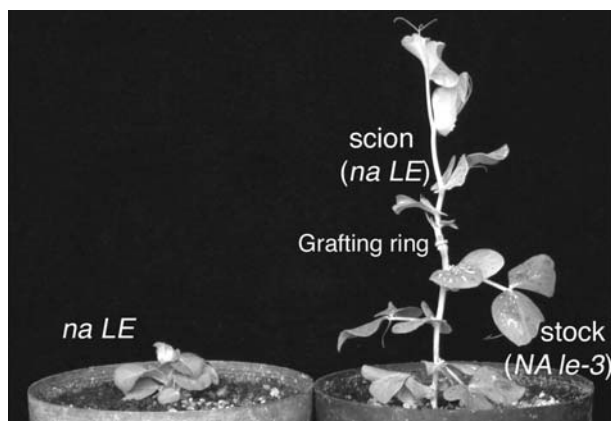
The developing seeds of many plant species typically contain higher levels of GAs, and a greater diversity of these compounds, than do vegetative tissues (MacMillan, 2001). Young seeds, in particular, often contain some unusual GAs and GA-related compounds; for example, young pea seeds contain substantial amounts of GA<sub>7</sub> dihydrodiol (Santes *et al.*, 1995), whereas this compound is much less abundant in other parts of the plant. On the other hand, older pea seeds (at the “table-ready” stage) tend to contain very high levels of a more limited number of GAs. This aspect, coupled with their large size, made older pea seeds ideal for early studies on GA biosynthesis (see Sponsel, 1995), and the sequence of steps in the early 13-hydroxylation pathway of GA biosynthesis was established using this system. Ironically, however, GAs do not appear to be important for seed development at these later stages, since the *ls-1* mutant, which blocks GA production early in the biosynthetic pathway (Swain *et al.*, 1997), dramatically reduces GA content in maturing seeds without affecting seed set or seed size (Swain *et al.*, 1995). GAs are critical at the early stages of seed development, however, since the *lh-2* mutation (which, like *ls-1* imposes a block early in GA biosynthesis; Davidson *et al.*, 2004) reduces bioactive GA levels in young seeds and results in substantial seed abortion, while the *lh-2* seeds that do develop are smaller than WT seeds (Swain *et al.*, 1993; 1995).

In earlier work it was suggested that seeds might act as sources of GAs for developing fruits (García-Martínez *et al.*, 1991). However, in pea, the *ls-1* mutant contains normal levels of the main bioactive GA, GA<sub>1</sub>, in young seeds, but is deficient in GA<sub>1</sub> in the pods (MacKenzie-Hose *et al.*, 1998). The same appears to be true for yet another early blocking mutation, *na-1* (Davidson *et al.*, 2003). The *na-1* mutation does not affect the GA content of maturing seeds, but dramatically reduces that of the pods (Potts, 1986). This indicates that in normal circumstances, the seeds do not export bioactive GAs (such as GA<sub>1</sub> and GA<sub>3</sub>) or their precursors into the elongating pods.

#### 9.4.2 *Vegetative tissues*

##### 9.4.2.1 *Grafting studies*

GA levels are generally much lower in vegetative tissues than in developing seeds (Sponsel, 1995; Ross *et al.*, 1995). However, the phenotypic effects of GA synthesis mutations are often more dramatic in the vegetative plant, and much early GA research addressed the related questions of GA distribution and transport at that developmental stage. It is well established that young, rapidly growing shoot tissue contains higher levels of bioactive GA than does fully mature tissue (Smith *et al.*, 1992; Ross *et al.*, 2003). However, where is that GA synthesised? With the isolation of



**Figure 9.5** Graft-transmissibility of  $GA_1$  precursors in pea. Stem and leaf growth in a *na-1* (GA-deficient) scion was promoted by grafting to a *NA* stock. The stock was itself deficient in  $GA_1$  (the bioactive GA), because of the presence of *le-3*, but it exported  $GA_1$  precursors into the scion where they were converted to  $GA_1$ . The plant on the left is an un-grafted *na-1 LE* plant.

dwarf, GA-deficient mutants, this question could be revisited, because in certain species, mutant and WT plants could be grafted together to determine whether or not GAs move from one genotype into the other. In pea, the first such grafting studies were performed with the *le-1* mutant that blocks the final step in  $GA_1$  biosynthesis (Ingram *et al.*, 1984). The dwarfing effect of this mutation was not graft-transmissible; that is, it could not be reversed by grafting to WT stocks (McComb & McComb, 1970). This result was confirmed by Reid *et al.* (1983), and by this time, the *na-1* mutant was also available (*na-1* blocks GA biosynthesis early in the pathway). Grafting *na-1* shoots (scions) to WT stocks (consisting of mature leaves and internodes) resulted in a dramatic promotion of elongation in the mutant (Reid *et al.*, 1983; Fig. 9.5). This provided evidence for the “long-distance” transport of a compound or compounds from one portion of the shoot (mature tissue) to another (immature tissue), where growth is stimulated. The *le-1*/WT and *na*/WT grafting results, taken together, indicate that a  $GA_1$  precursor, but not  $GA_1$  itself, is the mobile factor. It would appear that this precursor is a GA after  $GA_{12}$  in the pathway, since *na-1* blocks before  $GA_{12}$  (Davidson *et al.*, 2003). Other graft combinations showed that a major source of this mobile GA is mature shoot tissue, although a contribution from the roots cannot be ruled out (Reid *et al.*, 1983). Interestingly, although the *le-1*/WT grafts indicated that *endogenous*  $GA_1$  is not subject to long-distance transport in pea, it is well known that *applied*  $GA_1$  is at least partially transported from leaves to other parts of the plant (Ingram *et al.*, 1983; Steane *et al.*, 1989).

Further evidence that the endogenous mobile factor is a GA was provided by Proebsting *et al.* (1992), who showed that grafting *na* scions to WT rootstocks increased the actual GA content of the mutant scions. Proebsting *et al.* also presented evidence that the mobile GA is  $GA_{20}$ , and not  $GA_{19}$  (the precursor of  $GA_{20}$ ). The idea

of GA<sub>20</sub> as the mobile GA is consistent with the finding that in phloem diffusate from pea leaves, GA<sub>20</sub> was approximately 7-fold more abundant than GA<sub>19</sub> or GA<sub>1</sub> (García-Martínez *et al.*, 1991).

Grafting studies showed that GA<sub>1</sub> precursors can be exported from mature WT tissue to immature mutant tissue across a graft union, but does this mean that such transport normally plays a significant role in providing GAs to the young growing tissues? Grafting also showed that WT scions elongated to approximately the same extent whether grafted to *na* or WT mature stocks (Reid *et al.*, 1983), indicating that a supply of GA<sub>1</sub> precursors from the mature tissue is *not* necessary for the normal elongation of young internodes. However, it must be realised that as the WT scion develops, its own leaves expand and mature, and could be acting as sources of GA<sub>20</sub> for the elongating internodes (García-Martínez *et al.*, 1997). Thus, the extent to which elongating internodes rely on other tissues for GA<sub>20</sub> or other GA precursors is not yet known. It is relevant, however, that young internodes typically contain more GA<sub>19</sub> than GA<sub>20</sub> (Ross *et al.*, 2000), and this GA<sub>19</sub> might well have been synthesised *in situ*, since GA<sub>19</sub> might not be transported to a great extent (Proebsting *et al.*, 1992). Furthermore, the internodes themselves are capable of converting GA<sub>19</sub> to GA<sub>20</sub> (Smith, 1992; Jager *et al.*, 2005) and GA<sub>20</sub> to GA<sub>1</sub> (O'Neill & Ross, 2002). Therefore, there is considerable evidence that young internodes might be autonomous for GAs, and that they might not depend on imported GA precursors to maintain normal GA<sub>1</sub> levels.

#### 9.4.2.2 Can mature shoot tissue synthesise GAs?

During the 1990s, the sites of GA biosynthesis were also being studied using a different method. This approach relied on the logic that *ent*-kaurene, as the first committed intermediate in GA biosynthesis, represents a “gateway” into the GA biosynthesis pathway. On the basis of studying *ent*-kaurene biosynthesis, it was suggested (Moore & Coolbaugh, 1991; Aach *et al.*, 1995; 1997) that *ent*-kaurene synthesis, and therefore GA synthesis, is largely restricted to the rapidly expanding, immature shoot tissue. By implication, this research suggested that GA synthesis does not occur in mature tissue to any significant extent.

The Aach *et al.* (1995, 1997) articles were widely quoted, but the idea that GAs are not synthesised in mature tissue did not seem to fit with the grafting results, which indicated that mature shoot tissue can synthesise and export a GA<sub>1</sub> precursor, probably GA<sub>20</sub>, into other parts of the shoot. Therefore, Ross *et al.* (2003) reexamined the issue, by monitoring GAs in mature shoot tissue after excision of the immature material (decapitation). It was found that after decapitation, GA<sub>19</sub> and GA<sub>20</sub> levels were maintained in the mature tissue; in other words, it appeared that the GA<sub>19</sub> and GA<sub>20</sub> found in mature tissue was not coming from the immature region. Furthermore, evidence was obtained that these GAs were not coming from the roots (Ross *et al.*, 2003). The maintenance of normal GA<sub>19</sub> and GA<sub>20</sub> levels in decapitated plants did not seem to be due to slow metabolism of these GAs. Indeed, metabolism experiments with radiolabelled GAs indicated that mature tissue from both intact and decapitated plants rapidly converted GA<sub>19</sub> to GA<sub>20</sub>, GA<sub>20</sub> to GA<sub>1</sub>, GA<sub>1</sub> to GA<sub>8</sub>, GA<sub>20</sub> to GA<sub>29</sub> and GA<sub>29</sub> to GA<sub>29</sub>-catabolite.



Ross *et al.* (2003) concluded that in pea, mature tissues are able to synthesise GAs such as GA<sub>19</sub>, contrary to the suggestions of Aach *et al.* (1995, 1997). Nevertheless, there is little doubt that mature tissues contain much lower levels of the bioactive GA<sub>1</sub> than do immature tissues; in other words, there is co-localisation of GA<sub>1</sub> accumulation and rapid elongation in pea shoots. Ross *et al.* (2003) reported that the main reason for the steep gradient of GA<sub>1</sub> down the shoot is that mature tissues contain very strong GA<sub>1</sub> deactivation (2-oxidation) activity. GA<sub>20</sub> is also a substrate for 2-oxidation, and consequently GA<sub>20</sub> levels also decrease dramatically in older parts of the plant. GA<sub>19</sub>, on the other hand, is not 2-oxidised in pea, and mature tissue still contains substantial amounts of this GA. The end product of GA<sub>20</sub> 2-oxidation, GA<sub>29</sub>-catabolite, actually accumulates in the mature portion of the shoot (Ross *et al.*, 2003). In immature tissues, GA 2-oxidation activity is weaker, and GA<sub>1</sub> and GA<sub>20</sub> accumulate.

The conclusion that mature shoot tissue produces but rapidly deactivates GA<sub>1</sub> is supported by the expression pattern of a range of GA synthesis and deactivation genes. The synthesis genes *LS*, *LH*, *NA*, *PsGA20ox1* and *LE* (*PsGA3ox1*) are all strongly expressed in mature tissues, when the mRNA level is quantified in terms of the amount of mRNA per unit total RNA (García-Martínez *et al.*, 1997; Ross *et al.*, 2003; Davidson *et al.*, 2005). The GA deactivation (2-oxidation) gene *PsGA2ox1* (*SLN*) is also strongly expressed in mature shoot tissue (Elliott *et al.*, 2001; Ross *et al.*, 2003).

In potato, the theory that endogenous GA<sub>20</sub> is mobile, whereas endogenous GA<sub>1</sub> is not, would provide an explanation for the apparently anomalous tuberisation behaviour of certain transgenic potato plants (Prat, 2004). In this species, bioactive GAs in the stolons inhibit tuber formation (Jackson, 1999). It would be expected, therefore, that over-expression of GA synthesis genes would lead to an inhibition of tuber formation. In short days, this proved to be the case with a GA 20-oxidase gene, but not with a GA 3-oxidase. These genes encode enzymes for the steps GA<sub>19</sub> to GA<sub>20</sub> and GA<sub>20</sub> to GA<sub>1</sub>, respectively. Prat (2004) has suggested that endogenous GA<sub>20</sub>, but not endogenous GA<sub>1</sub>, is a mobile GA in potato (as in pea), and that when GA 3-oxidation occurs rapidly in the shoot (as in plants over-expressing a 3-oxidase gene), this consumes GA<sub>20</sub>, reducing the supply of GA<sub>20</sub> available for export from the shoot to the stolons. When a GA 20-oxidase gene is over-expressed, in contrast, GA<sub>20</sub> becomes abundant in the shoot, from where it can be transported to the stolons and converted to GA<sub>1</sub>, which inhibits tuber formation.

There is also evidence for the movement of endogenous GAs in germinating *Arabidopsis* seedlings. In this case, the suggestion is again that the precursors of bioactive GAs, rather than bioactive GAs themselves, are transported (Yamaguchi *et al.*, 2001). In *Arabidopsis* an early GA precursor, possibly *ent*-kaurene, is thought to move from the provascular tissue to the cortex and endodermis of the germinating seedling. The gene encoding the enzyme for *ent*-kaurene synthesis was expressed exclusively in the provascular tissue, while genes for later steps, including the step immediately after *ent*-kaurene, were expressed in the cortex/endodermis of embryo axes (Yamaguchi *et al.*, 2001).

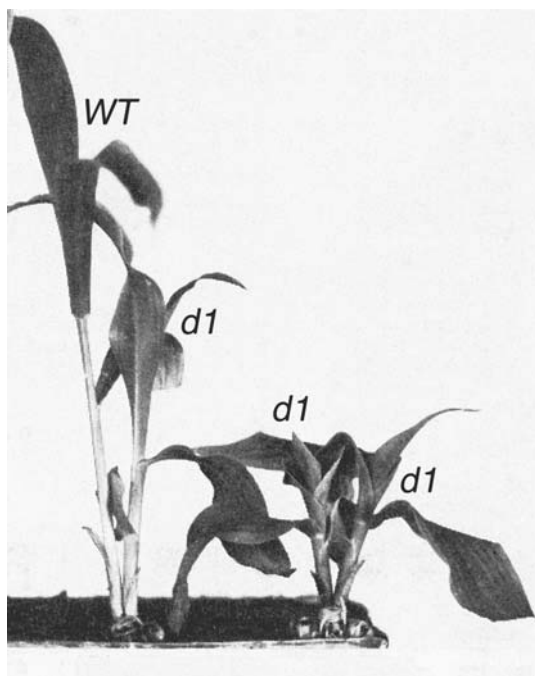
Interestingly, the mobility of *ent*-kaurene may not be limited to the plant body itself. It appears that *ent*-kaurene can be emitted from *Arabidopsis* plants over-expressing



*ent*-kaurene synthase (from a GA-producing fungus; Otsuka *et al.*, 2004). Furthermore, the airborne *ent*-kaurene then appeared to be taken up and converted to bioactive GAs by other *Arabidopsis* plants sharing the same container as the overproducers. Otsuka *et al.* (2004) also reported evidence that *ent*-kaurene is released by WT *Chamaecyparis obtusa* and *Cryptomeria japonica* plants, suggesting that emission of this GA intermediate may be a natural phenomenon. However, *ent*-kaurene was not released by seedlings of tomato, lettuce, pea or pumpkin (Otsuka *et al.*, 2004).

#### 9.4.2.3 Monocotyledonous species

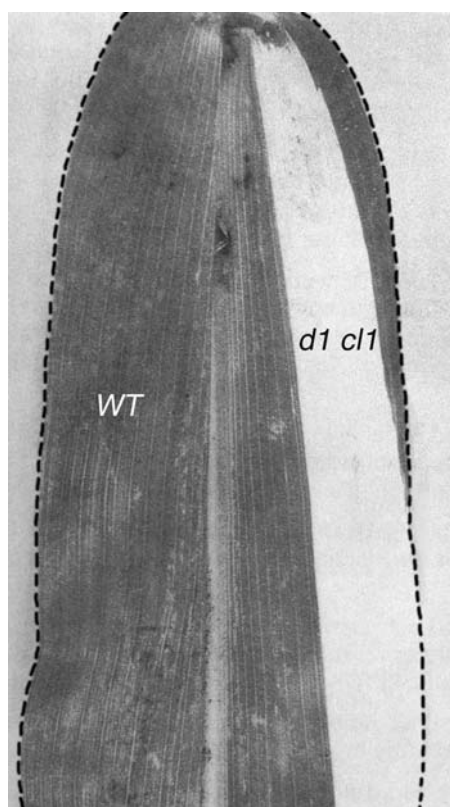
While the grafting studies in pea indicated that GA<sub>1</sub> is not transported, similar experiments in maize yielded a different result (Katsumi *et al.*, 1983). Katsumi *et al.* performed approach grafts, in which two seedlings were joined, but with each retaining its own root system. Two mutants were used: *d*<sub>5</sub> and *d*<sub>1</sub>, which impose early and late blocks on the GA biosynthesis pathway, respectively. Similar to the *le-1* mutation from pea, the *d*<sub>1</sub> mutation is defective in the step from GA<sub>20</sub> to GA<sub>1</sub> (Spray *et al.*, 1984). The key results were that grafting *d*<sub>1</sub> plants to the WT promoted elongation in the mutant (Fig. 9.6), and that grafting *d*<sub>5</sub> to *d*<sub>1</sub> promoted elongation in the *d*<sub>5</sub> partner.



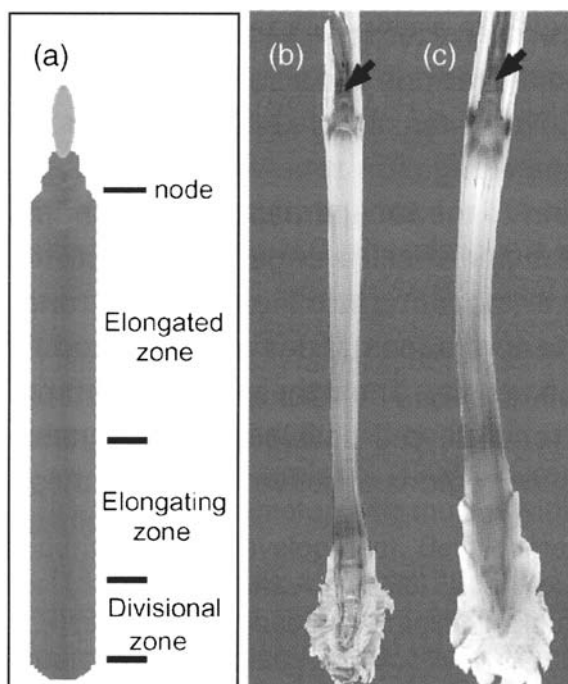
**Figure 9.6** Graft-transmissibility of GA<sub>1</sub> in maize. Shoot elongation in a *d*<sub>1</sub> (GA<sub>1</sub>-deficient) shoot was promoted by grafting to a WT shoot (left). The graft on the right consists of two *d*<sub>1</sub> shoots grafted together. In these approach grafts both partners continue to grow (reproduced with permission from Katsumi *et al.*, 1983, Oxford University Press).

These findings indicate that both  $GA_1$  and its precursors are transported in maize, in contrast to pea where there is only evidence for transport of the precursors.

Using a different approach, Winkler and Freeling (1994) also obtained evidence that endogenous  $GA_1$  is transported in maize. These workers created a mosaic plant consisting of WT leaves with sectors of genotype  $d_1$ . The  $d_1$  sectors, which comprised up to 45% of the distance from mid-vein to leaf margin, were immediately recognisable because they also carried  $cl$ , which conferred whiteness (lack of chlorophyll) to the tissue. The reasoning was that if  $GA_1$  did not move from the WT section of the leaf, where it was produced, the mosaic leaves would become distorted as they grew, because  $d_1$  sections, lacking  $GA_1$ , would elongate to a lesser extent (by approximately 50%). However, no such distortion was observed (Fig. 9.7), and the elongation of the mutant sectors appeared to be normal. These



**Figure 9.7** Evidence for the movement of  $GA_1$  in maize. The  $d_1$  sector (white) is deficient in  $GA_1$  biosynthesis but still elongated normally when adjacent to WT tissue. The overall elongation of the leaf was not distorted by the presence of the sector. The white colour resulted from chlorophyll deficiency caused by the  $cl$  allele, also carried by the  $d_1$  sector (reproduced with permission from Winkler & Freeling, 1994, Oxford University Press). The leaf margins are shown by dotted lines.



**Figure 9.8** Co-localisation of growth and expression of GA biosynthesis genes in rice. Left panel: (a) the elongating fifth internode can be divided into three parts, from the bottom to the top: divisional zone, elongating zone and elongated zone. Right panel: the expression of two GA biosynthesis genes from late in the pathway is greater in the divisional–elongating zones than in the elongated zone. Shown is the localisation of  $\beta$ -glucuronidase ( $\beta$ -GUS) activity under the control of the promoter of the (b) *OsGA3ox2* gene (step  $GA_{20}$  to  $GA_1$ ) or (c) the *OsGA20ox2* gene (step  $GA_{19}$  to  $GA_{20}$ ) (modified from Kaneko *et al.*, 2003).

observations indicate that a mobile factor, probably  $GA_1$  itself, moved from WT tissues into the  $d_1$  sections, promoting elongation.

It is possible, therefore, that there is a difference between monocotyledonous and dicotyledonous plants with respect to the potential of endogenous  $GA_1$  to be transported. However, even though endogenous  $GA_1$  appears *capable* of being transported in monocotyledonous species, we need to again ask: in these species, does the transport of  $GA_1$  and/or its precursors normally make a substantial contribution to the  $GA_1$  pool at sites where GA acts? In other words, is there, or is there not, co-localisation of the sites of GA synthesis and GA action? This question was addressed for rice by Kaneko *et al.* (2003), who monitored the expression of GA biosynthesis and signaling genes in various plant parts at several stages of development (Fig. 9.8). On this basis Kaneko *et al.* (2003) suggested that at most developmental stages, bioactive GA is synthesised (from its immediate precursors) in the same zone in which it acts. For example, in 2-week-old seedlings, the synthesis and signaling genes were all expressed in young leaves surrounding the SAM,

whereas little or no activity was detected in the developed leaves. However, since the genes monitored were from late in the GA pathway, the gene-expression data on their own do not preclude the possibility that *early* GA intermediates move from another part of the seedling to the young leaves enclosing the SAM.

In grasses, the major exception to the co-localisation pattern reported by Kaneko *et al.* (2003) involves the germinating grains, where there is clear spatial separation between GA synthesis and GA action (Woodger *et al.*, 2004). In germinating cereal grains GA<sub>1</sub> is transported from the embryo (specifically, the epithelium of the embryo) to the aleurone layer, where it stimulates the production and secretion of  $\alpha$ -amylases and other hydrolases. In support of this classical model, Kaneko *et al.* (2003) found evidence for the expression of GA signaling genes, but not GA synthesis genes, in the aleurone layer. Earlier experiments indicated also that GAs were synthesised in the embryo (Lenton *et al.*, 1994) rather than in the aleurone.

Another situation in which a GA is thought to be a critical mobile signal in grasses relates to flowering in *Lolium temulentum*. In this species one long day, perceived by the leaves, is sufficient to promote flowering at the shoot apex, and it is suggested that the communicating signal is GA<sub>5</sub>, which possesses strong florigenic activity in this species (King & Evans, 2003).

#### 9.4.3 Conclusion: some GAs can undergo long-distance transport, at least in some circumstances

In summary, for dicots there is evidence that in certain circumstances (e.g. in grafts), GA precursors can move from mature tissues into the elongating zone, where they are converted to bioactive GAs that in turn promote elongation growth. In the grasses, there is evidence for the potential mobility not only of GA precursors, but of the bioactive species as well. However, the contribution that transport normally makes to the GA pool of rapidly growing tissues is still not known, but what is clear is that the highest levels of bioactive GA are usually found in zones of rapid growth. Nevertheless, the co-localisation of bioactive GA and rapid growth (Sponsel & Hedden, 2004) should not be confused with the co-localisation of GA *biosynthesis* and growth. There is little doubt that mature pea shoot tissue, well past the growth stage, can still synthesise GAs. That bioactive GA does not accumulate in mature pea tissue is attributable to rapid GA deactivation.

## 9.5 BRs: distribution and transport

The third hormone group to be discussed are the BRs. The BRs are steroidal compounds now known to be essential for normal plant growth and development (Clouse, 2002). Extensive research over the past two decades has revealed the importance of BRs in a wide variety of processes, including cell elongation, cell division, vascular differentiation, reproductive development, and pathogen and abiotic tolerance (Clouse, 2002). As a consequence, this group is now widely

recognised as an important class of plant hormones, alongside the “classical” hormones such as auxin and GA.

### 9.5.1 *BR distribution*

BRs were originally isolated from pollen (Mitchell *et al.*, 1970) which, along with other reproductive organs such as seeds, generally contains the highest concentration of these substances (Bajguz & Tretyn, 2003). With the development of techniques for the analysis of endogenous BR levels and the expression of BR biosynthesis genes, researchers have recently been able to address specific questions regarding the distribution of BRs in other plant tissues. Such studies have revealed clear spatial patterns of distribution of BRs in a wide range of plant species (Bancos *et al.*, 2002; Shimada *et al.*, 2003; Symons & Reid, 2004). For instance, Bancos *et al.* (2002) showed that BRs were present in both the shoot and root tissues of pea, *Arabidopsis* and tomato plants. However, the levels of the early intermediates in the BR biosynthesis pathway were generally higher in the roots, while the late-pathway BRs, castasterone (CS) and 6-DeoxoCS, were more abundant in the shoots (Bancos *et al.*, 2002). In 2003, Shimada *et al.* published a comprehensive report on both endogenous BR levels and BR biosynthesis gene expression, in a wide range of different tissues from *Arabidopsis* seedlings. This study demonstrated a widespread distribution of endogenous BRs throughout the plant, with the greatest accumulation of late-pathway BRs occurring in the young actively growing tissues. Significantly, there was a close association between the tissues that show the highest BR concentrations and those that showed the highest expression levels of BR biosynthesis genes (Shimada *et al.*, 2003). Symons and Reid (2004) have demonstrated a similar distribution pattern of BR levels in pea.

The occurrence of the highest BR levels in the young, actively growing tissues is consistent with a key role for these hormones in plant development. Given the importance of BR we would expect the existence of mechanisms that strictly control endogenous BR levels and their distribution in the target cells or tissues. As is the case for other hormones, such mechanisms could include the relative rates of BR synthesis, destruction, inactivation and transport within the plant. The close association between BR levels and the expression of BR biosynthesis genes (Shimada *et al.*, 2003) suggests that *in situ* BR biosynthesis may be one such mechanism that plays a significant role in regulating the endogenous BR levels. However, the widespread distribution of endogenous BRs also raises the possibility that long-distance transport of these compounds may occur between different plant tissues, and that this may also play a role in regulating localised BR levels.

### 9.5.2 *BR transport*

#### 9.5.2.1 *Exogenous BRs*

Early attempts to understand BR transport involved monitoring the movement of radiolabelled BRs after application to the plant (previously reviewed by Arteca,

1995; Adam & Schneider, 1999; Sasse, 1999; 2003; Bishop & Yokota, 2001). Such studies provided some evidence that BRs may be transported acropetally from the roots to the shoots. For instance, a small percentage of [ $^3\text{H}$ ]brassinolide (BL) or [ $^3\text{H}$ ]castasterone (CS) applied to the roots of rice plants was reported to be translocated to the shoots (Yokota *et al.*, 1992). Similarly, when [ $^{14}\text{C}$ ]epiBL was applied to the roots of cucumber and wheat seedlings  $^{14}\text{C}$  was soon detected throughout both plant species (Nishikawa *et al.*, 1994).

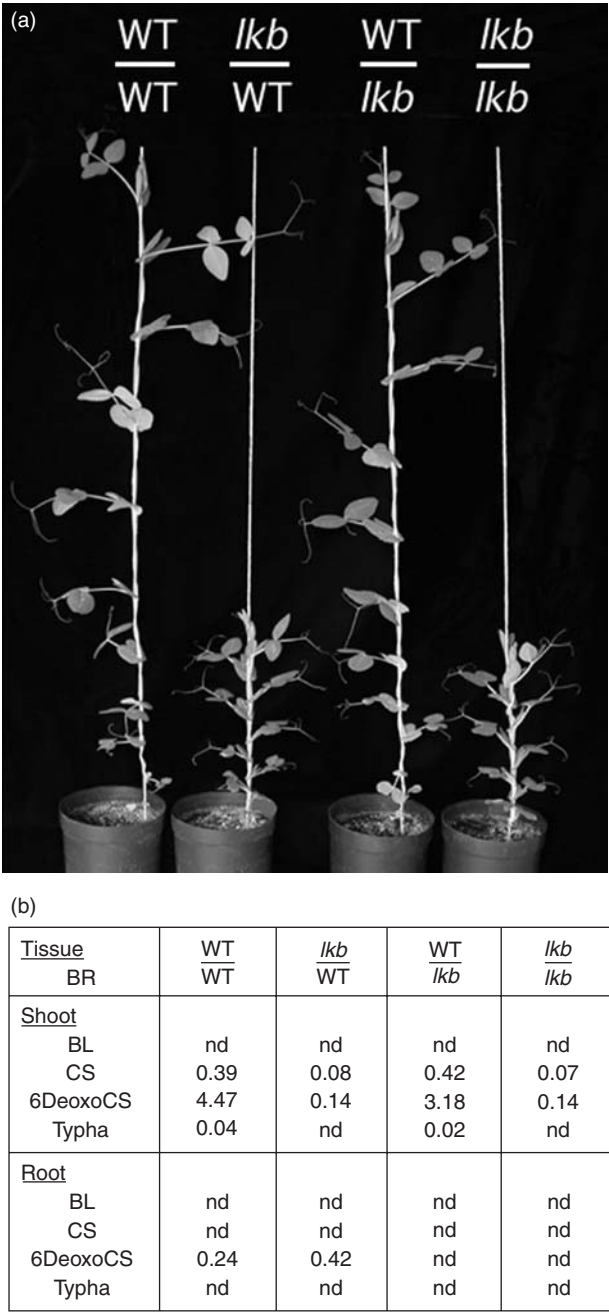
In contrast to BRs applied to plant roots, exogenous BRs applied to shoot tissues of different species have proven to be relatively immobile. For instance, Symons and Reid (2004) showed that [ $^3\text{H}$ ]BL and [ $^3\text{H}$ ]CS applied to the shoot tissues of pea entered the plant, but did not move beyond the site of application. These results are consistent with previous studies, which show that the majority of radiolabelled BL and CS incorporated into leaves of rice remained in the treated leaves 24 h after it was applied (Yokota *et al.*, 1992). In this study a small amount of radioactivity was shown to accumulate in the roots after 72 h, but this was largely in the form of water-soluble BR metabolites (Yokota *et al.*, 1992). Similarly, in wheat, exogenously applied [ $^{14}\text{C}$ ]epiBL was not transported from the treated leaf even after 7 days (Nishikawa *et al.*, 1994).

Despite these relatively clear outcomes, we must be cautious in our interpretation, because the observed movement of exogenous radiolabelled substances may not accurately reflect transport of endogenous hormones (Hoad, 1995). Therefore, while some of these studies demonstrate that *exogenous* BRs *can* move acropetally from the roots, they do not necessarily prove that *endogenous* BRs are normally transported in a similar manner. It is inherently difficult to make definitive conclusions about hormone transport on the basis of hormone application studies alone.

#### 9.5.2.2 *Endogenous BRs: grafting studies*

As was the case for the GAs, grafting studies have provided invaluable insights into the transport of *endogenous* BRs in plants. Initial grafting studies using the pea *lkb* mutant (Reid & Ross, 1989) were conducted prior to the discovery that the *lkb* mutation causes BR deficiency, by blocking BR biosynthesis (Nomura *et al.*, 1999; Schultz *et al.*, 2001). In this initial study, young *lkb* scions were grafted onto mature leafy WT stocks, but the presence of this WT root and shoot tissue did not restore internode elongation in the *lkb* scion (Reid & Ross, 1989). With the benefit of hindsight we now know that this result indicates that *endogenous* bioactive BRs are not transported acropetally (upwards) in pea shoots (Bishop & Yokota, 2001). However, these initial studies revealed little about the possibility of basipetal transport within the shoot (e.g. from the apical bud to the internodes), or from the shoot to the root.

Armed with techniques for the quantification of endogenous BR levels, Symons and Reid (2004) revisited grafting studies using WT and *lkb* mutant plants. Significantly, this study revealed further compelling evidence that *endogenous*, bioactive BRs do not undergo long-distance transport. For example, it was confirmed that a WT rootstock does not restore the growth of a BR-deficient *lkb* shoot (Fig. 9.9; Reid & Ross, 1989), and the reason was shown to be that bioactive BR levels in the



**Figure 9.9** Evidence that BRs are not graft-transmissible in pea. Reciprocal grafting between WT and *lkb* mutant plants did not affect the (a) phenotype of the shoot nor (b) endogenous BR levels in shoot and root tissues. The grafts were made epicotyl to epicotyl using 7-day-old seedlings (reproduced with permission from Symons & Reid, 2004, copyright ASPB).



*lkb* shoot are not increased by the presence of the WT rootstock (Symons & Reid, 2004). Assuming that grafting does not disrupt normal BR transport pathways, these results provide further evidence that the maintenance of normal bioactive BR levels in the pea shoot is not dependent on acropetal BR transport from the roots (Symons & Reid, 2004).

In the reciprocal graft combination (a WT shoot grafted onto an *lkb* rootstock), the presence of the WT shoot did not increase BR levels in the BR-deficient *lkb* root (Fig. 9.9; Symons & Reid, 2004). Once again, if we assume that grafting does not disrupt normal BR transport pathways, this result suggests that the maintenance of normal bioactive BR levels in pea roots is not dependent on basipetal BR transport from the shoots (Symons & Reid, 2004).

Comparable grafting studies in tomato, using WT and the BR-deficient, extreme dwarf  $d^x$  mutant plants, have also yielded similar results, with no phenotypic recovery of the  $d^x$  phenotype in either reciprocal graft combination (Bishop *et al.*, 1999; Montoya *et al.*, 2005). These results reinforce the conclusion that *endogenous* BRs do not undergo long-distance transport between shoots and roots, and show that this phenomenon is consistent in different plant species. Therefore, it is likely that the acropetal movement of *exogenously* applied BRs from the roots (Yokota *et al.*, 1992; Nishikawa *et al.*, 1994) may not accurately reflect the transport of *endogenous* BRs in plants.

#### 9.5.2.3 BR transport within the shoot?

While the grafting studies clearly show a lack of BR transport (acropetal or basipetal) between shoot and root, they provide only limited information regarding the possible transport of BRs within these tissues. This issue was also addressed by Symons and Reid (2004), who examined the effects of decapitation (removal of the apical bud) and defoliation (removal of leaves) on endogenous BR levels in the shoot. In this case, neither decapitation nor defoliation resulted in a decrease in endogenous BR levels in the remaining shoot tissues. This suggests that the maintenance of normal BR levels in the stem is not dependent on BR transport from either the apical bud or the mature leaves. Furthermore, the maintenance of normal BR levels in the mature leaves does not depend on BR transport from the apical bud and *vice versa* (Symons & Reid, 2004).

Other evidence that BRs do not move over long distances (from one organ to another) within plant shoots has come from characterisation of the BR-deficient  $d^x$  mutant of tomato (Bishop *et al.*, 1996). In this study several transposon-induced  $d^x$  mutant lines were shown to exhibit a variegated phenotype, consisting of revertant WT sectors within shoots that otherwise exhibited a  $d^x$  mutant phenotype (Fig. 9.10; Bishop *et al.*, 1996). The fact that these WT sectors (presumably with normal BR levels) exist within a  $d^x$  background, without restoring the  $d^x$  phenotype, indicates that BRs are not freely diffusible around the shoot. Such results are also consistent with the apparent immobility of *exogenous* BRs when applied to the shoot (Yokota *et al.*, 1992; Nishikawa *et al.*, 1994; Symons & Reid, 2004).



**Figure 9.10** Evidence that endogenous BRs are not transported within the leaf. The variegated tomato leaf shown consists of BR-deficient mutant leaflets (wrinkled) and revertant leaflets that are WT in appearance because they produce BRs. The BRs produced by the revertant leaflets do not appear to move into the mutant leaflets (modified from Bishop *et al.*, 1996).

#### 9.5.2.4 “Short-distance” BR transport?

While many of the studies discussed above rule out long-distance transport of bioactive BRs between the shoot and roots, and between different organs within the shoot, they do not exclude the possibility that BRs may move short distances within or between plant cells. Indeed, a closer examination of the phenotype of the transposon-induced  $d^x$  mutant lines (Bishop *et al.*, 1996) provides some evidence that such short-distance BR transport may occur in plants. For instance, the boundaries between the  $d^x$  mutant tissue and revertant (WT) sectors were reported to be diffuse, and were found mainly at physical boundaries such as the veins (Bishop *et al.*, 1996). Based on this observation it could be suggested that BRs might move over a short distance (i.e. cell to cell, within the leaflet), a scenario in which a small number of genotypically revertant cells would be sufficient to generate comparably large phenotypically revertant sectors (Bishop *et al.*, 1996).

A potential mechanism for BR transport in plants has been proposed by Markovic-Housley *et al.* (2003). This involves binding of BRs to a specific pathogenesis-related protein (PR-10) to form a complex that would allow the relatively apolar BRs to be transported from the cytosol to their receptors. It has been suggested that this mechanism may be of crucial importance in the plant defence response to pathological situations as well as in plant growth and development (Markovic-Housley *et al.*, 2003). Clearly the possible existence of short-distance or micro-scale BR transport, the mechanism by which this occurs, and its potential role in the regulation of BR levels and plant development are all worthy of further investigation.

### 9.5.3 Conclusion: endogenous BRs do not undergo long-distance transport

The weight of evidence clearly suggests that endogenous BRs do not undergo long-distance transport between the shoot and root or between different organs within the shoot. While short-distance or micro-scale BR transport remains a possibility, it appears that endogenous BR levels are regulated primarily through site-specific control of BR biosynthesis, catabolism and conjugation.

## 9.6 General discussion

This chapter has highlighted some significant similarities and differences regarding the distribution and transport of three of the main growth-promoting hormones that have been discovered and characterised so far. All three (IAA, GAs and BRs) occur at higher levels in young, rapidly expanding tissues than in more mature parts of the shoot, although in pea, the steepness of this gradient down the stem is greatest for GA<sub>1</sub> and the least for the BRs, with IAA intermediate. All three hormones can be detected in mature shoot tissue, and – although GA<sub>1</sub> levels are very low in that case – there seems little doubt that mature shoot tissue can synthesise GA precursors.

It is when we turn to hormone transport that major differences between the hormones emerge. Plants appear to have evolved a specialised transport system for only one of the three: auxin. Certain GAs may well be transported, but there is no evidence that this occurs in any system other than the plant's normal vasculature (probably the phloem). There is good evidence that in some circumstances precursors of bioactive GAs (in dicotyledonous species) or possibly the bioactive GAs themselves (in grasses) are transported, although it is not clear at present what contribution this makes to the GA pool in young, rapidly growing tissues. It may well be the case that elongating internodes can both synthesise GA precursors and convert these to the bioactive form, given the right conditions. As far as the BRs are concerned, there is no definitive evidence at present for transport from one plant part to another.

The auxin transport system allows the plant to develop auxin gradients, which in turn convey positional information. The discovery that auxin dramatically affects GA biosynthesis (Ross *et al.*, 2000; 2001) helps us to understand how this can then affect development and why apparently only one of the hormones requires a specialised transport system. Auxin may well promote elongation, at least in stems, by upregulating the level of active GA (Ross *et al.*, 2000). According to this model, auxin is the mobile factor that communicates between the apical bud and the elongating internodes, and bioactive GA is the ultimate hormonal effector of stem elongation growth, synthesised in the internodes themselves under the influence of auxin imported from above. In this way a single specialised transport system influences the level not only of the hormone actually transported, but of a second hormone as well.

The classification of plant-growth-regulatory substances as “hormones” has, on occasions, created conceptual problems by implying a similarity to animal endocrine

systems (Davies, 1995). This has led to the assumption that a plant hormone must be synthesised by one organ before being transported and perceived by other tissues (Bishop & Yokota, 2001). Although this may be true for auxin, this scenario does not seem to apply to the BRs: it seems likely that BRs are synthesised and act in the same tissues, or perhaps even in the same cells. Therefore, not all growth-promoting hormones meet the “transport” criterion regarding their status as plant “hormones”. Nevertheless, the recent characterisation of mechanisms controlling perception and signal transduction of auxin, GAs and BRs (Li & Chory, 1997; Wang *et al.*, 2001 for BRs; Dharmasiri *et al.*, 2005 for auxin; Kepinski & Leyser, 2005; Ueguchi-Tanaka *et al.*, 2005 for GAs; and additional chapters in this volume) leaves no doubt about the vital importance of these signaling compounds in the control of plant growth and development.

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## 10 Reproductive development

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### 10.1 Introduction

Similar to other essential processes in plant growth, reproductive development consists of a highly regulated series of decisions that control the timing, the spatial distribution and the morphology of newly arising organs. Hormones are an integral part of this signaling network, and there are multiple examples to support the view that the participation of hormones in these regulatory events provides plasticity and robustness in the response to environmental and endogenous cues. In this chapter, we will review and discuss our current knowledge on how the different hormones affect the diverse aspects of reproductive development, with emphasis in the molecular aspects of the mechanisms involved.

### 10.2 Flowering time

The transition from vegetative growth to reproductive development is a complex but well-organized process that involves changes in gene expression, physiology, metabolism and architecture of the plant (Bernier & Périlleux, 2005). The transition between these developmental programmes is executed through a change in the nature of the shoot apical meristem, transforming leaf-into-flower-type primordia (Cary *et al.*, 2002; Jacqmard *et al.*, 2003). These events are tightly regulated and the resulting onset of flowering is controlled by endogenous cues and environmental factors. Both endogenous and exogenous factors regulate flowering time through different well-characterized pathways. The main environmental factors controlling flowering are light and temperature, and the corresponding inputs are driven through the photoperiod-dependent, vernalization and thermosensory pathways (Blázquez *et al.*, 2003; Hayama & Coupland, 2003; Sung & Amasino, 2004). In addition, endogenous cues, including age- or size-related factors and the activity of hormones and carbohydrate-related metabolites, also control the transition to flowering. These factors exert their regulation through the autonomous and the gibberellin (GA)-dependent pathways (King & Evans, 2003; Simpson, 2004). There are also multiple points of connection between the flowering pathways, for example, the low-temperature effect of vernalization is based on endogenous epigenetic effects, mediated by the degree of genomic DNA methylation (Sung & Amasino, 2004). In this section, we will focus on the effect of classical and more recently discovered phytohormones on the transition to flowering.

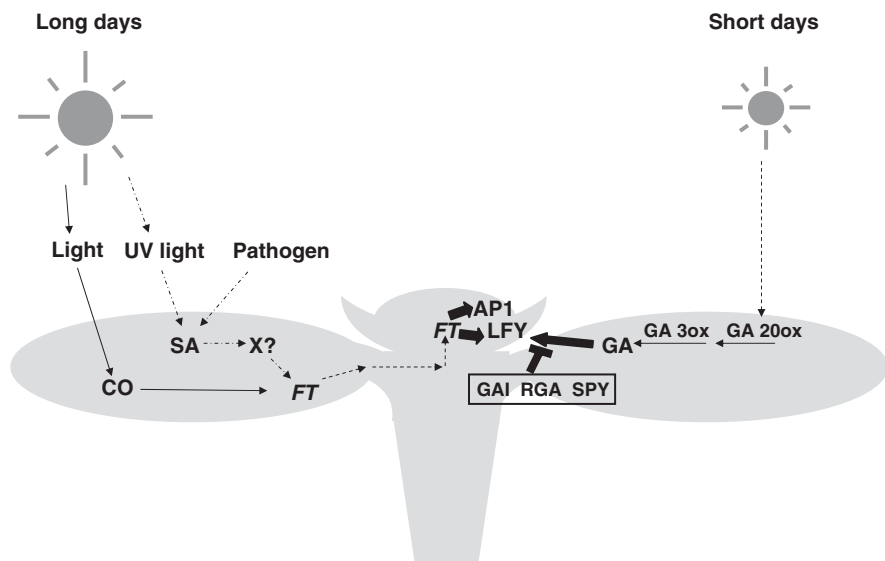
### 10.2.1 Gibberellins

The molecular mechanisms involved in GA signaling in flowering and other developmental processes have been recently and extensively reviewed (King & Evans, 2003; Sun & Gubler, 2004).

Physiological and genetic data have supported GAs as hormones that accelerate flowering in the model plant *Arabidopsis* and in other plants. The application of physiologically active GAs to plants leads to early flowering in *Arabidopsis* (Langridge, 1957) and other plants such as *Lolium temulentum*, *Larix laricina* and *Pinus sylvestris* (King *et al.*, 1993; Eysteinnsson & Greenwood, 1995). Moreover, the GA content increased in the shoots of plants induced to flower (King *et al.*, 2001). Regulation by GAs may result not only from changes in synthesis, translocation and accumulation but also from sensitivity to the hormone (Oka *et al.*, 2001). In the absence of environmental factors that promote flowering, *Arabidopsis* maintains the transition to the reproductive phase via the GA pathway. This is supported by the lack of flowering of the GA-deficient mutant *gal* under photoperiodic conditions of day-length shorter than 10 h (Wilson *et al.*, 1992). Moreover, overexpression of GA biosynthesis genes or activation of GA signaling in the *spindly* (*spy*) mutant result in early flowering (Jacobsen & Olszewski, 1993; Coles *et al.*, 1999). The GA-dependent pathway to flowering requires the function of the products of genes such as *GAI* (Wilson *et al.*, 1992), *GAI*, *RGA* (Dill & Sun, 2001), *FPL1* (Kania *et al.*, 1997), *SPY* (Tseng *et al.*, 2004) and possibly *GAMYBs* (Gocal *et al.*, 2001). The signaling pathway that drives GA-dependent transition to flowering may be regulated by the micro-RNA miR159, which seems to function by cleaving the mRNA encoding the *GAMYB*-related proteins (Achard *et al.*, 2004) and hence, by preventing the GA-induced activation of the floral meristem identity gene *LEAFY* (*LFY*) in *Arabidopsis* (Blázquez *et al.*, 1998). In contrast, the rice *GAMYB* gene does not seem to regulate the transition to flowering but the development of floral organs (Kaneko *et al.*, 2004). A similar role in anther development has also been attributed to the *Arabidopsis* *GAMYb33* and *GAMYb65* genes, whose expression is restricted to anthers in part by the action of miR159 (Millar & Gubler, 2005).

Although GAs are essential for flowering of *Arabidopsis* growing under short-, but not long-day conditions, there is evidence to ascribe to GA a role in the promotion of photoperiod-induced flowering. For instance, in *L. temulentum*, the application of certain GA molecules is as efficient as a single long-day pulse to induce flowering (Evans *et al.*, 1990), GAs applied to intact leaves are transported to the apex and promote flowering (King *et al.*, 2001), and long-day treatments induce a two-fold increase in GA content in the apex (King *et al.*, 2003). Besides, a functional connection between GAs and the long-day pathway could occur via negative interaction between *SPY* and the *GIGANTEA* protein thought to act in the photoperiod pathway (Tseng *et al.*, 2004). However, the activation of flowering through the *Arabidopsis* *LFY* gene is regulated independently by GAs and the long-day pathway (Blázquez & Weigel, 2000; Blázquez *et al.*, 2002; Fig. 10.1).





**Figure 10.1** Integration of hormone action in the network that controls flowering time in *Arabidopsis*. GAs are essential for flowering under short-day conditions, mainly through their effect upon *LFY* expression. SA mediates the promotion of flowering by stress conditions through the regulation of *FT*, which in turn activates the expression of meristem identity genes in the apex. SA: salicylic acid.

### 10.2.2 Brassinosteroids

It has been recently reported that *BAS1* and *SOB7* genes coding for cytochrome P450s in *Arabidopsis* modulate photomorphogenesis through brassinosteroid (BR) inactivation mechanisms (Turk *et al.*, 2005). Both genes act redundantly in such a way that the double *bas1 sob7* mutant displays an early flowering phenotype (Turk *et al.*, 2005). The positive regulation on flowering time exerted by BRs indicated by these data is in agreement with the late flowering phenotype reported for the *det2* mutant affected in BRs biosynthesis (Chory *et al.*, 1991) and the delayed bolting reported for the *bls1* (brassinosteroid, light and sugar 1) mutant involved in regulating endogenous BR levels (Laxmi *et al.*, 2004). In contrast, other mutations in previously characterized BR-specific biosynthesis genes *DWF1*, *DWF4*, *CPD*, *BR6ox1* and *ROT3* do not seem to affect the timing of the transition from vegetative to reproductive stage, probably because of BR homeostasis mechanisms based on feedback control of the expression of genes involved in its metabolism (Tanaka *et al.*, 2005).

### 10.2.3 Auxins, cytokinins and ethylene

Auxins have been extensively characterized as hormones with essential regulatory functions in root and shoot development. They act by a strict control of transport and gradient accumulation patterns (Kepinski & Leyser, 2005). Many components of the

auxin signaling response have been identified and characterized including a number of Auxin/indole-3 acetic acid (Aux/IAA) inhibitors and auxin response factor (ARF) transcription factors that seem to yield specificity in regulating different developmental processes through a combinatorial strategy (Weijers & Jurgens, 2004; see also Chapter 2). Despite the relevant function of auxins in plant development, their regulatory role on the control of the transition to flowering in plants is somehow secondary or indirect. It has been reported that auxins in association with cytokinins, ethylene or abscisic acid (ABA) may modulate flowering time as suggested by the delayed flowering phenotype of some *Arabidopsis* mutants such as *hyl1* (hyponastic leaves 1) or *alh1* (ACC-related long hypocotyl 1), which are affected in sensitivity to auxin, cytokinin, ABA or ethylene, respectively (Lu & Fedoroff, 2000; Vandenbussche *et al.*, 2003). Also a regulatory connection between auxins and GAs has been proposed in the control of morphogenesis in pea plants. The late flowering phenotype of the *nal* pea mutant seems to be explained by a defect in auxin regulation of GA biosynthesis (Demason, 2005). Also recently, it has been reported that mutations in the *ARF2* gene confer delayed flowering to different mutant *arf2* alleles in *Arabidopsis* (Okushima *et al.*, 2005).

Cytokinins regulate many vegetative developmental processes (Howell *et al.*, 2003). Despite physiological evidence reported about cytokinin implication in the control of flowering time (Bernier *et al.*, 1993), there is no genetic evidence supporting a role for this hormone in controlling the transition from vegetative to reproductive development.

#### 10.2.4 Abscisic acid

ABA regulates many growth and developmental processes and is especially relevant in regulating water- and osmotic-related stress (Himmelbach *et al.*, 2003; see also Chapter 1). Most of the ABA-response mutants described display only subtle phenotypic defects in the absence of stress. In contrast, the *ABA insensitive 8* (*abi8*) mutant displays severe developmental defects including delayed flowering (Broccard-Gifford *et al.*, 2004). Moreover, the *ABA hypersensitive 1* (*abh1*) recessive mutation suppresses the late flowering phenotype conferred by certain *FRIGIDA* (*FRI*) and *FLC* alleles in *Arabidopsis* due to the inability of *FRI* to increase *FLC* mRNA levels (Bezerra *et al.*, 2004). Although an interpretation of these observations is that the effect of ABA on flowering may be indirect, the identification of the classical flowering time regulator *FCA* as an ABA receptor provides a direct link between this hormone and the onset of reproductive development, possibly as a response to stress conditions (Razem *et al.*, 2006). However, ABA may regulate flowering also through *FCA*-independent mechanisms since the double mutants *abi1 fca1* and *abi2 fca1* flowered earlier than *fca1* plants (Chandler *et al.*, 2000).

#### 10.2.5 Salicylic acid and the stress-activated transition to flowering

The onset of flowering is tightly regulated by interactions between different pathways controlling flowering time. As a result, plants flower at the appropriate time

of year to ensure efficient reproduction. However, this tight control may be overridden in stressed plants and premature flowering may occur. A variety of stress factors including pathogen infection, drought, extreme light irradiation and temperatures have been reported to induce accelerated flowering in different plants (Fox, 1990; Stanton *et al.*, 2000; Veronese *et al.*, 2003). Some of these stress factors, such as biotrophic pathogen infection or irradiation with ultraviolet C (UV-C) light, cause increases in the endogenous salicylic acid (SA) content (Yalpani *et al.*, 1994; Dempsey *et al.*, 1999; see also Chapter 8). Although a flowering promoter activity was assigned to SA in reports published in the 1970s and 1980s (Cleland, 1974; Cleland & Ajami, 1974; Goto, 1981), no information on the mechanisms by which SA modulates flowering time was available. It has been recently reported that UV light irradiation, which induces SA production, can accelerate flowering through a SA-dependent mechanism, whereas mutations that confer SA-deficiency lead to late flowering in *Arabidopsis* (Martínez *et al.*, 2004). The SA-regulated transition to flowering seems to be the result of interactions with the photoperiod-dependent and autonomous pathways but is independent of the functions of flowering time *CO* and *FCA* genes (Martínez *et al.*, 2004). In any case, evidence points to a regulatory role of SA upon the expression of *FT*, whose RNA is transported from the leaves to the apex in order to activate floral meristem identity genes (Huang *et al.*, 2005; Fig. 10.1)

### 10.3 Flower development

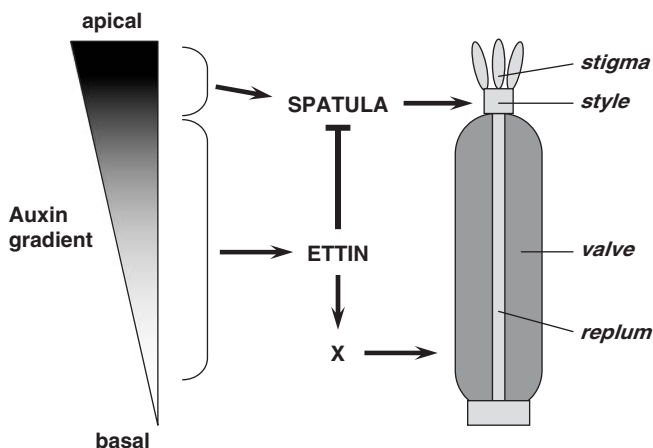
Although aberrant morphologies of flowers have been reported after the application of several hormones and biosynthesis inhibitors, only two hormones have been unequivocally associated with flower morphogenesis and are likely to have a regulatory role in the differentiation that occurs during flower formation. GAs have been implicated in the control of floral homoeotic gene expression and anther development, while auxin gradients seem to regulate ovary development and organ initiation, as we will summarize below.

A very clear hint of the involvement of GA in flower development is the male sterility observed in *Arabidopsis* GA-deficient *gal* mutants (Koornneef & van der Veen, 1980), in which microsporogenesis occurs, but pollen grains are not viable. An equivalent defect is observed in the GA-deficient *d5* mutant of maize (Jacobsen & Olszewski, 1991) and in petunia plants treated with the GA biosynthesis inhibitor paclobutrazol (Izhaki *et al.*, 2002), which formed anthers loaded with pollen grains, but the connective tissue and the tapetum were degenerated. Moreover, the tomato GA-deficient *gib-2* mutant is arrested in an earlier developmental stage, prior to microsporogenesis, and is also sterile (Nester & Zeevaart, 1988; Jacobsen & Olszewski, 1991), similarly to the *myb33 myb65* double mutant in *Arabidopsis*, in which microsporogenesis is dramatically affected and the tapetum enlarged and eventually collapses (Millar & Gubler, 2005). However, GAs not only regulate late differentiation stages of anther and pollen development, but a deficiency of GAs in

*Arabidopsis* causes retarded growth of petals, stamens and pistils, which can be rescued by knocking out negative elements of the GA signaling pathway expressed during flower development, such as *RGA* and *RGL2* (Tyler *et al.*, 2004; Yu *et al.*, 2004). However, fertility of the *gal* mutant is not fully recovered even after simultaneous elimination of *RGA*, *RGL1* and *RGL2*, which is in agreement with a possible role for also *RGL3* in pollen development (Tyler *et al.*, 2004). In this context, GAs seem to be necessary to promote the expression of the floral homeotic genes *APETALA3*, *PISTILLATA* and *AGAMOUS*, because *gal* mutants show lower expression levels of these genes, and a glucocorticoid-inducible form of *RGA* can repress their expression after dexamethasone treatments of nascent flowers (Yu *et al.*, 2004). The physiological relevance of this regulation is further supported by the observation that overexpression of *AGAMOUS* partially suppresses the floral defects of the *gal* mutant (Yu *et al.*, 2004).

Finally, there is an additional role for GAs in later stages of flower differentiation. It has been shown that GAs produced in anthers are transported to the corolla and induce growth and pigmentation. This latter effect can be attributed to the promotion of anthocyanin synthesis in petunia petals by GAs (Weiss *et al.*, 1992; Weiss *et al.*, 1995; Weiss, 2000), which requires the coordinated upregulation of at least 15 genes that encode enzymes involved in flavonoid biosynthesis, including chalcone synthase.

An essential role for auxin in the generation of new floral organs is based on multiple observations. First, genetic evidence implicates auxin flux in the determination of floral organ number and patterning. Mutations in *PIN-FORMED 1* (*PIN1*), a gene that encodes an auxin efflux carrier, result in only few flowers being formed and multiple structural abnormalities, including a decreased number (and reduced size) of sepals, petals and stamens, and a marked loss of valve tissue in the ovary (Okada *et al.*, 1991). This effect is phenocopied by applications of the auxin polar transport inhibitor *N*-1-naphthylphthalamic acid (NPA). Part of this effect may be attributed to the role of auxin in general organ outgrowth, as suggested by the observation that petunia *floozy* mutants, defective in a flavin mono-oxygenase required for auxin biosynthesis, do not show alterations in floral organ identity but the initiation of organ primordia is impaired at an early stage (Tobena-Santamaría *et al.*, 2002). However, the defects in the specification of valve tissue in the gynoecium point to a more specific role for auxin gradients in the specifying patterning of the ovary (Fig. 10.2). This is particularly evident when interpreting the equivalent valve defect caused by mutations in *MONOPTEROS* (Przemeck *et al.*, 1996; Hardtke & Berleth, 1998) and *ETTIN* (*ETT*; Nemhauser *et al.*, 2000), the genes encoding ARF5 and ARF3, respectively, two transcription factors that participate in auxin signaling (Kepinski & Leyser, 2005). In the current model, *ETT* would establish two boundaries in the ovary to mark the apical and basal ends of the valves, by responding to intermediate concentrations in an apical–basal gradient of auxin, and directing valve tissue differentiation (Nemhauser *et al.*, 2000). Much of the role of *ETT* in valve differentiation would actually be to prevent downregulation of the style-specific gene *SPATULA* in valve tissue (Heisler *et al.*, 2001), for which it might require the activity of additional proteins such as SEUSS (Pfluger & Zambryski, 2004).



**Figure 10.2** *Involvement of auxin in the patterning of the Arabidopsis gynoecium.* Intermediate auxin levels in an apical–basal gradient would act through the auxin-response factor ETTIN to induce valve differentiation and prevent SPATULA from invading valve tissue.

## 10.4 Early fruit development

Hormones have been used for a long time to induce fruit set in an agronomical context in many diverse species (Nitsch, 1970; Schwabe & Mills, 1981; Gillapsy *et al.*, 1993). In many occasions, hormone applications tend to increase fruit yield, but they are also used to induce the formation of seedless fruits. Both effects may for instance be observed in certain citrus species and cultivars (Talón *et al.*, 1990a, b), and are also important in tomato, in which certain unfavourable environmental conditions reduce pollen production and thus fruit set (Gustafson, 1936). From a basic research perspective, these observations have led to the hypothesis that in these treatments, the effect of the applied hormones reflects a role for the endogenous hormones in the early events during the formation of fruits. However, there have been two alternative views of this phenomenon: one in which hormones trigger early fruit development upon pollination by activating the differentiation programme, and another one in which hormones are required only to direct nutrient resources to the differentiating fruit. Cytokinins have been reported to induce parthenocarp when applied to unpollinated pea ovaries (Eeuwens & Schwabe, 1975) and *Arabidopsis* (Vivian-Smith & Koltunow, 1999), but correlative and genetic evidence for the implication of auxin, GAs and polyamines in early fruit development is stronger. Although it is not possible to propose an unifying model for the involvement of the different hormones in the diverse types of fruits (fleshy fruits, pods, siliques, etc.), certain trends are common for all the species studied, as will be shown below.

### 10.4.1 Gibberellins

The first systematic studies in which a biochemical approaches was used to dissect the mechanism by which GAs regulate early fruit development were in pea.

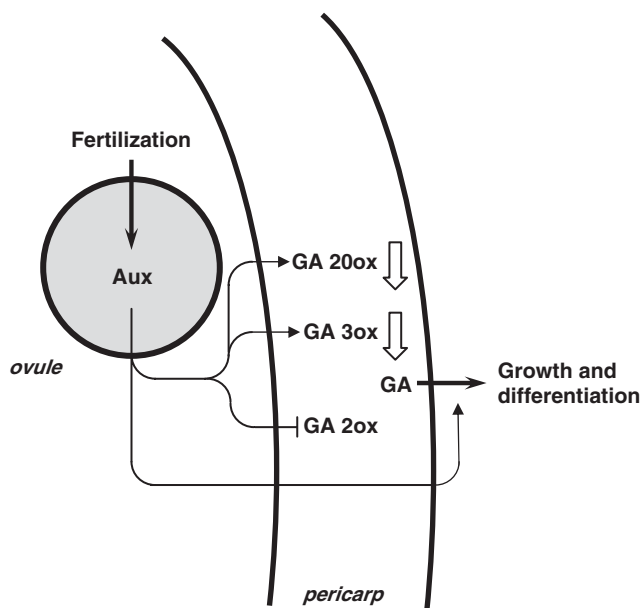
Unpollinated pea ovaries, as in many other species, are programmed to senesce unless the appropriate stimulus is provided – generally ovule fertilization, but also treatments with GAs (García-Martínez & Carbonell, 1980). This system has been very useful to evaluate rapid changes in hormone levels following fertilization, and the conclusion is that GA biosynthesis is increased as a consequence of successful pollination (García-Martínez *et al.*, 1991), through the upregulation in the pericarp of the expression of genes encoding enzymes involved in GA biosynthesis (García-Martínez *et al.*, 1997; Ozga *et al.*, 2002). Work with *Arabidopsis* has provided the genetic evidence that supports previous physiological and molecular studies, since mutants impaired in GA biosynthesis or signaling have been found to show defects in fruit initiation and growth (Barendse *et al.*, 1986; Vivian-Smith & Koltunow, 1999).

The involvement of GAs in early fruit development is not restricted to pods and siliques. Pollination in citrus causes a very clear increase in GA levels (Ben-Cheikh *et al.*, 1997) and, more interestingly, citrus varieties that naturally produce seedless fruits (such as *Satsuma* or *Clementina mandarins*) have increased GA levels that correlate with the differential ability of the species tested (Talón *et al.*, 1990a, b). This latter correlation has also been observed in fleshy fruits of tomato. For instance, the ovaries of several tomato *pat* mutants (parthenocarpic) have a higher GA content, and parthenocarpy is abolished with paclobutrazol (Fos *et al.*, 2000; Fos *et al.*, 2001). However, the *pat-1* mutants have defects in ovule and anther development (Mazzucato *et al.*, 1998) so, at this point, it is still difficult to assess if this alteration of GA metabolism is in fact the primary cause of the observed parthenocarpy.

#### 10.4.2 Auxin

Auxin treatments are very effective in inducing fruit set in certain species, particularly tomato (Gustafson, 1936), but also *Arabidopsis* (Vivian-Smith & Koltunow, 1999). Parthenocarpy in tomato has been associated with the accumulation of auxins in the ovaries (George *et al.*, 1984), and it is generally accepted that the contribution of auxin is more important than that of GAs for early fruit development in tomato (Bangerth, 1981). However, it has been reported that auxin derived from the apical shoot seems to prevent fruit development in the absence of the appropriate stimuli (i.e. fertilization) (Rodrigo & García-Martínez, 1998; Rodrigo *et al.*, 1998) thus complicating the interpretation of the overall role of auxin in fruit formation.

Ovules are indeed a major source for auxin (Archbold & Dennis, 1985), and localized upregulation of auxin biosynthesis in ovules might trigger early fruit development, as supported by the generation of parthenocarpy in transgenic tomatoes and eggplants that overproduce auxin in ovules (Rotino *et al.*, 1997). A possible mechanism for the initiation of fruit development might thus involve production of auxin in the developing seeds, and then activation of GA biosynthesis by auxin, which would direct growth and differentiation of the fruit tissue (Fig. 10.3). This model is supported by observed upregulation of GA biosynthesis genes in pea pericarp by



**Figure 10.3** Possible mechanism for the interaction between auxin and GAs during early fruit development. This model summarizes our knowledge in different plant species, and is based in a fertilization-induced increase in auxin in the ovule, which in turn promotes the upregulation of GA biosynthesis in the walls of the ovary. GAs and auxin then direct tissue differentiation and growth.

4-chloro-IAA, resulting in increased GA concentration (van Huizen *et al.*, 1995; 1997; Ngo *et al.*, 2002; Ozga *et al.*, 2003; Ross *et al.*, 2000).

#### 10.4.3 Polyamines

There is varied evidence supporting a role for polyamines during early fruit development (Evans & Malmberg, 1989; Egea-Cortines & Mizrahi, 1991). For example, the activity of arginine decarboxylase, the enzyme that initiates polyamine biosynthesis, decreases at the end of ovary development, but a transient increase is observed after fruit set induced by GA treatments in unpollinated ovaries (Pérez-Amador & Carbonell, 1995; Pérez-Amador *et al.*, 1995), consistent with the idea that polyamines are required for growth in general. This is supported by the observation that application of polyamines can partially promote fruit development in unpollinated tomato ovaries (Fos *et al.*, 2003), and that the concentration of endogenous polyamines is rapidly altered in tomato fruits after pollination (Egea-Cortines *et al.*, 1993). Notably, there is a correlation between high spermine level and ovary senescence, and between low spermine level and fruit development induced by pollination, auxin or GAs in pea (Carbonell & Navarro, 1989) and in tomato (Alabadí *et al.*, 1996). This might mean that spermine is part of the signals that induce senescence in unpollinated ovaries, and pollination would reduce spermine levels (via GA and possibly



auxin). Nevertheless, this model lacks the support of genetic evidence, since the available mutants defective in spermine biosynthesis in *Arabidopsis* have not been evaluated for fruit set or ovary senescence (Imai *et al.*, 2004).

## 10.5 Fruit maturation

Fruit maturation is a complex biochemical and physiological process that involves numerous changes in fruit attributes including organoleptic qualities, nutritional content and susceptibility to opportunistic pathogens. The process is regulated by endogenous and external factors that include developmental gene regulation, hormones, light and temperature through a coordinated network of signaling pathways (Adams-Phillips *et al.*, 2004a). Fruit ripening varies among different species but all kinds of fruits mature essentially either in a climacteric or non-climacteric way depending on whether increased respiration and ethylene synthesis is required or not to complete the process (Giovannoni, 2001). Most of the progress in studying fruit maturation comes from the combination of three experimental systems: *Arabidopsis* as a model plant with dry fruits, and two fleshy fruit plants, tomato, as a model for climacteric fruits, and strawberry, as a model for non-climacteric fruits (White, 2002).

### 10.5.1 Ethylene

There is no doubt that ethylene is the best-known factor regulating fruit ripening. A vast amount of information coming from physiological approaches in different experimental systems supports this regulatory role, and the biosynthesis and action of ethylene in tomato is likely the best-studied experimental system in climacteric fruit maturation (Alexander & Grierson, 2002). However, from the molecular point of view, most of the advances come from molecular-genetic studies on the perception and biosynthesis machinery of ethylene in *Arabidopsis* (Etheridge *et al.*, 2005). Perception of ethylene in *Arabidopsis* is mediated by the function of five receptors (Chang & Stadler, 2001) that act as redundant negative regulators of ethylene signaling (Hua & Meyerowitz, 1998). In tomato, six ethylene receptors have been identified and characterized (Klee & Tieman, 2002), and the genes encoding two of them, *NEVER-RIPE (NR)* and *LeETR4*, are strongly induced at the onset of ripening (Tieman & Klee, 1999); however, their respective loss of function have opposite effects on tomato fruit ripening (Wilkinson *et al.*, 1995; Tieman *et al.*, 2000). Downstream of the receptors, the putative MAP-kinase *CTR1* seems to act, by interacting with receptors, as a negative regulator of ethylene signaling in *Arabidopsis* (Gao *et al.*, 2003). Several *CTR1* homologous genes have been identified in tomato and other species and they show differential expression patterns, at least one of them being induced during ripening (Adams-Phillips *et al.*, 2004b). Downstream of *CTR1* in *Arabidopsis*, the sequential function of *EIN2*, *EIN3* and *EIL* gene products connect to the function of the *ERF1* transcription factor that regulate

the ethylene signaling via binding to GCC-box promoter elements of target genes (Etheridge *et al.*, 2005). Homologues of *Arabidopsis* *EIN3*, *EIL* and *ERF* have also been identified in tomato as multigene families with at least a member of each family exhibiting ripening-induced expression (Tournier *et al.*, 2003; Yokotani *et al.*, 2003).

In non-climacteric citrus fruit, although ethylene production increases in young fruitlets, this hormone does not play an essential role in the ripening of mature fruits (Katz *et al.*, 2004). On the other hand, a cDNA microarray-based analysis of gene expression in ripening non-climacteric strawberry fruits allowed the identification of several ethylene signaling-related genes with patterns of expression associated with achene maturation (Aharoni & O'Connell, 2002). Moreover, it has been recently reported that different ethylene receptors show increased expression during ripening of strawberry fruits coinciding with the production of a small amount of ethylene, which suggests that such a small increase may be sufficient to trigger ripening (Trainotti *et al.*, 2005).

### 10.5.2 Auxin

Exogenous application of synthetic auxins to different plant species either hastens or delays ripening (Davies *et al.*, 1997; Payasi *et al.*, 2004). Thus, it is controversial whether endogenous auxins act as positive or negative regulators of fruit ripening. Several Aux/IAA and ARF transcription factors have been reported to be coordinately regulated by ethylene and auxins during tomato fruit maturation. In fact, one of these genes, called *DR12*, belonging to the ARF family of transcription factors seems to play a role in tomato fruit ripening, as transgenic plants underexpressing the *DR12* gene displayed several defects including dark-green immature fruits and blotchy ripening (Jones *et al.*, 2002).

It has been reported that the decline in auxin levels supplied from the achenes to the receptacle during fruit maturation was associated with the onset of ripening in a non-climacteric fruit such as strawberry (Given *et al.*, 1988). Microarray-based analysis of ripening strawberry fruits led to the identification of many auxin-repressed genes involved in different processes of fruit maturation such as pigmentation, cell wall metabolism and flavour/aroma synthesis (Aharoni *et al.*, 2002).

### 10.5.3 BRs and ABA

The application of BRs to tomato pericarp discs led to accelerated ripening through a process that seems to be mediated by ethylene biosynthesis (Vardhini & Rao, 2002). Moreover, the BR biosynthetic mutant *det2* displayed a reduced expression of an endo-1,4- $\beta$ -D-glucanase likely involved in the assembly of cellulose-hemicellulose network in the cell wall during ripening (Nicol *et al.*, 1998).

Also ABA seems to play a role in promoting banana fruit ripening through, at least in part, ethylene-mediated mechanisms (Jiang *et al.*, 2000). In ripening avocado fruits the rise in ethylene production precedes an increase in ABA biosynthesis from carotenoid cleavage (Chernys & Zeveaart, 2000).

### 10.5.4 Salicylic acid

The involvement of SA in the regulation of fruit ripening is somewhat controversial. It has been reported that treatment of banana fruits with SA delayed ripening (Srivastava & Dwivedi, 2000). However, the tomato calcium-dependent protein kinase *LeCRK1* is induced during fruit ripening as well as in SA- or ethylene-treated leaves, and its expression is undetectable in natural tomato ripening mutants such as *NR*, *Rin* and *Nor* (Leclercq *et al.*, 2005). The function of SA during ripening may well be related to regulation of resistance to fungal pathogens in mature fruits. In fact, interaction between the anthracnose fungus, *Colletotrichum gloeosporioides*, with pepper fruits varies from incompatibility with ripe fruits to compatibility with unripe fruits. Regarding this, a *PepThi* gene, which is pathogen inducible, is also activated by SA in unripe fruits (Oh *et al.*, 1999). Another likely function of SA during ripening of climacteric fruits could be the regulation of ethylene production through modulation of the expression of ethylene biosynthetic genes. A wound- and ripening-induced 1-aminocyclopropane-1-carboxylate (*ACC*) synthase gene from tomato is inhibited by SA (Li *et al.*, 1992).

## 10.6 Conclusions

As seen above, hormones impinge on multiple pathways that regulate reproductive development. The challenge for the next few years is the elucidation of the molecular mechanisms by which this regulation occurs, so that direct and indirect effects for particular growth regulators can be distinguished. Acquiring this detailed level of knowledge seems particularly relevant if a long-term goal is to be able to fine tune-specific aspects of reproductive development without affecting the general architecture of the plants or other growth patterns.

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# **11 Seed development and germination**

Shinjiro Yamaguchi and Eiji Nambara

## **11.1 Introduction**

In higher plants, seed dormancy and germination are complex physiological processes that are influenced by many genetic and environmental factors. Many lines of genetic, physiological and biochemical evidence have illustrated the importance of two plant hormones, abscisic acid (ABA) and gibberellin (GA), in the regulation of seed dormancy and germination. Antagonistic actions of ABA and GA during seed development and germination have been thought to be achieved through alterations in sensitivities and/or endogenous levels of these hormones. In addition to the roles of ABA and GA, there is emerging evidence to suggest some roles of other hormones in controlling seed germination.

During the course of seed development, the embryo remains attached to the mother plant for a long period of time, even after it completes morphogenesis and acquires the ability to germinate. This association with the mother plant enables the seed to accumulate reserves, acquire desiccation tolerance and establish dormancy. When the mature seed are dispersed and then imbibed, the seed have to decide “to stay dormant” or “to germinate”. This decision is determined by sensing various environmental factors, such as light, temperature and nutrients (Bewley, 1997a). These external signals often alter metabolisms and sensitivities of plant hormones. In particular, the antagonistic actions of ABA and GAs are known to be an important determinant for seed germination. In this review, we summarize recent advances in understanding the regulation of hormone metabolisms and actions, especially ABA and GA, both of which are tightly linked to developmental and environmental factors. We also discuss the crosstalk of ABA, GA and other hormones during seed development and germination.

## **11.2 Hormonal control of seed development**

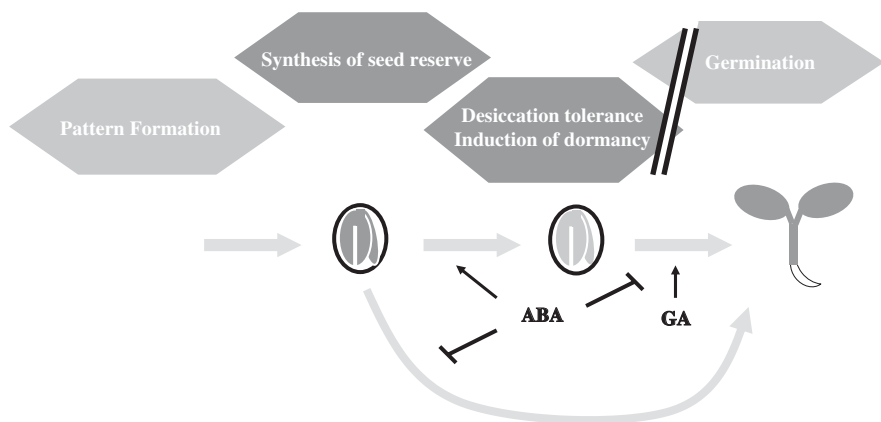
Recent molecular genetic analyses have revealed that the actions of ABA and GAs are modulated directly by developmental regulators, which are known to define “space” or “timing” of embryo development. Importantly, these regulators act as a “balancer” of the metabolism and sensitivities of these two hormones that in part explains the antagonistic actions of ABA and GAs. These findings also suggest that the hormone itself plays an indispensable role in regulating developmental processes more deeply than we have thought. In this section, we first introduce developmental phases and regulators for seed development, and then summarize

current knowledge on the interaction of ABA and GAs in terms of both metabolism and signaling.

### 11.2.1 Developmental and physiological phases in seed development

Developmental and physiological phases are defined by expression of phase-specific genes, thus the phase transition requires both down-regulation of genes for the former phase and up-regulation of genes for the latter phase. Galau (1987) and his colleagues isolated a large number of genes that are differentially expressed during seed development and germination in cotton (*Gossypium hirsutum*) (Galau *et al.*, 1987; Hughes & Galau, 1989). They defined five phases in seed development and germination of dicotyledonous plants by the marker gene expression as well as by the timing of physiological and morphological processes (Galau *et al.*, 1991). Recent molecular genetics and transcriptome analyses from various plants suggest that these phases are similar in angiosperms (Rensink & Buell, 2005).

In general, developmental processes in seed development are largely divided into two phases: pattern formation (morphogenesis) and seed maturation. The seed maturation is further divided physiologically into two phases: based on before or after abscission, here designated as mid maturation stage and late embryogenesis, respectively (Fig. 11.1). The mid maturation stage is characterized by expression of genes for seed reserve synthesis especially seed storage protein genes, while the late embryogenesis by the expression of late embryogenesis abundant (Lea) protein genes. Lea proteins are hydrophilic proteins and are structurally divided into at



**Figure 11.1** Phases of seed development. Seed development can be divided into several phases. Three developmental and physiological phases are shown here. An initial phase is characterized as the morphological process (pattern formation). The second phase, maturation stage, is defined by expression of seed storage protein genes and by ABA accumulation. In this stage, cell division and differentiation ceased. The third phase, late embryo development or post-abscission stage, is associated with abscission and is defined by the expression of Lea genes. Desiccation tolerance and induction of dormancy occurred at this stage.

least six groups (Wise & Tunnacliffe, 2004). Although their specific function is still unclear, several lines of evidence indicate that they are involved in the acquisition of desiccation tolerance, which occurs in late embryogenesis.

Mature dry seed contains a large amount of mRNAs that is thought to be utilized after imbibition. The presence of these transcripts was illustrated by Nakabayashi *et al.* (2005) using microarray analysis on dry and imbibed *Arabidopsis* seeds. This study, using oligonucleotide-based microarrays representing approximately 22,000 genes, revealed that more than 10,000 mRNA species were detected both in dry and imbibed seeds. Furthermore, it was demonstrated that proximally located genes are often co-regulated in imbibed seeds; thus, the up-regulated genes or down-regulated genes constitute 3 to 5 gene “expression clusters” on the chromosome. This co-regulation of gene expression is consistent with the finding that the genes for stored mRNAs expressed at high levels are often located in tandem on the chromosome.

Rajjou *et al.* (2004) reported that *Arabidopsis* seeds can germinate in the presence of  $\alpha$ -amanitin, an inhibitor of polIII-mediated transcription, although it is delayed compared to the untreated controls (Rajjou *et al.*, 2004). In contrast, the treatment of seeds with a translational inhibitor, cycloheximide, abolishes germination completely. Somewhat surprisingly, a subset of proteins is newly synthesized prior to germination even when the transcription is blocked. These proteins include seed storage proteins and Lea proteins, for which mRNAs are abundant in dry seed. These findings suggest that both stored mRNAs and *de novo* synthesized mRNAs are necessary to initiate early events after seed imbibition and allow successful germination.

Plant developmental and physiological phases are loosely separated, such that the phase transitions occur gradually (Poethig, 1990). Consequently, there is considerable overlap between the processes of late embryogenesis and germination. In cotton, oilseed (*Brassica napus*), and *Arabidopsis*, a subset of germination-associated genes is expressed in late embryogenesis (Harada *et al.*, 1988; Hughes & Galau, 1989; Nambara *et al.*, 2000). Conversely, a subset of transcripts for late embryogenesis-associated genes is present during seed germination. The heterochronic mutations in *Arabidopsis* alter the timing of such gene expression: precocious expression of the latter (germination-associated) genes and down-regulation of gene expression for maturation and late embryogenesis. Notably, these heterochronic mutations render phase transition more gradual, suggesting that multiple checkpoints exist between the phases (Nambara *et al.*, 2000).

### 11.2.2 Developmental regulators for seed development

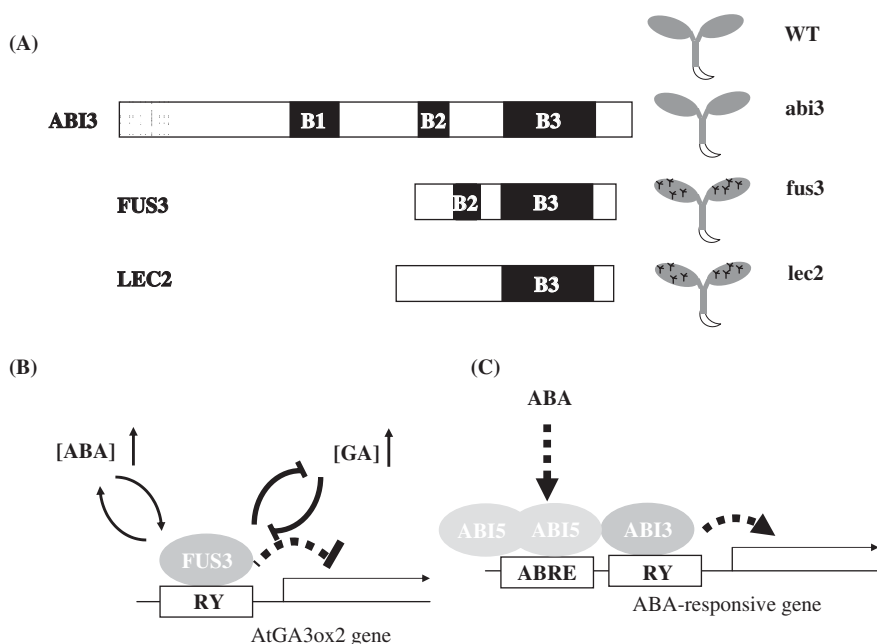
Defects in the regulators of maturation stage and late embryogenesis lead to reduced accumulation of seed reserves, improper acquisition of seed dormancy and desiccation intolerance. These mutants often exhibit precocious germination on the mother plants and produce desiccation intolerant seeds (McCarty, 1995). They are also conditional lethal, but can be rescued when immature seeds are harvested and sown prior to desiccation. Such non-dormant mutants are reported in various plant

species, especially *Arabidopsis* and maize. In *Arabidopsis*, four transcription factors (*LEAFY COTYLEDON 1 (LEC1)*, *LEAFY COTYLEDON 2 (LEC2)*, *FUSCA 3 (FUS3)* and *ABSCISIC ACID-INSENSITIVE 3 (ABI3)*) have been identified genetically as important developmental regulators of seed development (Giraudat *et al.*, 1992; Lotan *et al.*, 1998; Luerben *et al.*, 1998; Stone *et al.*, 2001). In maize, many mutations that result in vivipary occur in the genes necessary for the biosynthesis of ABA or its intermediate carotenoids (McCarty, 1995). In addition, the *VIVIPAROUS 1 (VP1)* gene encodes a transcription factor that is the orthologue of ABI3

Consistent with their role as regulators of seed development, the *Arabidopsis lec1*, *fus3*, *lec2* and *abi3* mutants exhibit severe defects in the maturation stage and late embryogenesis (Meinke *et al.*, 1994). These mutants produce desiccation intolerant seeds with a reduced amount of seed reserves. The *LEC1*, *FUS3*, *LEC2* and *ABI3* genes are essentially considered to be specific regulators of seed development and germination, because the mutants do not exhibit dramatic developmental defects in vegetative and reproductive growth. The *abi3* mutant also demonstrates a strong resistance to ABA-mediated inhibition of seed germination, indicating a role in regulating ABA sensitivity (Nambara *et al.*, 1992; Ooms *et al.*, 1993). In contrast, mutants of the *LEC1*, *FUS3* and *LEC2* loci show relatively normal sensitivity to ABA, although the *lec1* mutant shows reduced sensitivity to ABA (Parcy *et al.*, 1997). A common defect of the *lec1*, *lec2* and *fus3* mutants is the presence of trichomes on their cotyledons. In *Arabidopsis*, wild-type plants form trichomes only on true leaves, but not on cotyledons (Fig. 11.2a). Such a phenotype in these mutants is interpreted as the result of a loss of cotyledon identity.

*LEC1* encodes a HAP3 subunit of a CCAAT-box-binding factor that is widely conserved among eukaryote transcription factors (Lotan *et al.*, 1998). The CCAAT-box is postulated to be involved in the expression of a particular set of co-regulated genes, such as nitrogen metabolism and non-fermentable carbon-inducible genes in yeast and cell-cycle-regulated genes in higher eukaryotes. The expression pattern of the *LEC1* is seed specific, which probably reflects a specific role in seed development (Lotan *et al.*, 1998). It is interesting to note that ectopic expression of the *LEC1* transforms the true leaves into cotyledon-like organs, indicating that *LEC1* is sufficient to confer the cotyledon identity (Lotan *et al.*, 1998).

The maize *VP1* gene was shown to encode a transcription factor that contains a conserved B3 basic domain (McCarty *et al.*, 1991). In addition to the B3 domain, VP1 has three other conserved domains: one acidic domain (A1) and two basic domains (B1 and B2). The truncated VP1 protein, containing the B3 domain, binds specifically to an Sph element including the seed-specific enhancer RY repeat (Suzuki *et al.*, 1997), which is highly conserved in the promoters of seed protein genes of both monocots and dicots. However, the B3-deleted *vp1* allele still retains some residual function in seed development, indicating that VP1 can also function via the B3-independent manner. The *Arabidopsis ABI3* gene was shown to be an orthologue of *VP1* (Fig. 11.2a) (Giraudat *et al.*, 1992). Ectopic expression of the *ABI3* can induce the expression of seed protein genes in vegetative tissues of transgenic *Arabidopsis* plants, when exogenous ABA is applied to these plants (Parcy *et al.*, 1994). This



**Figure 11.2** Comparison of structures and functions of B3 transcription factors. (a) (Left panel) *ABI3* and *VP1* orthologues contain four conserved domains: A1, B1, B2 and B3. The B1 domain is required for the interaction with *ABI5* (or *TRAB1*). *FUS3* and *LEC2* are relatively small proteins that lack B1 domains. The B3 domain is required for the binding to RY elements (Right panel). Loss-of-function of *FUS3* or *LEC1* causes ectopic formation of trichomes on the cotyledons that are observed normally in vegetative organs. (b) *FUS3* acts by balancing metabolism of ABA and GA. In seed development, *FUS3* acts positively on ABA accumulation, whereas it acts negatively on bioactive GA synthesis. Both ABA and GA levels influence *FUS3* levels as indicated in the figure. *FUS3* balances ABA and GA metabolism through positive and negative regulatory loops (connecting with ABA and GA, respectively). The negative role of *FUS3* in GA biosynthesis is, at least in part, derived from the repression of *AtGA3ox3* expression through direct binding to its RY element(s). (c) *ABI3* is the node of ABA signaling and developmental programs. ABA signaling elicits ABRE-mediated transcription. During seed development, the *ABI5* bZIP transcription factor is responsible mainly for ABRE-mediated transcription through direct binding. This response is enhanced by *ABI3* through its binding to RY.

indicates that *ABI3* is sufficient to elicit seed-specific expression. In addition to *ABI3/VP1*, both *FUS3* and *LEC2* encode B3 domain-containing proteins (Luerben *et al.*, 1998; Stone *et al.*, 2001). However, they do not contain the conserved B1 domain (Fig. 11.2a). The B3 domains of the *ABI3* and *FUS3* are structurally similar and both transcription factors bind to the RY repeat *in vitro* (Curaba *et al.*, 2004; Mönke *et al.*, 2004). Ectopic expression of *FUS3* or *LEC2* in transgenic plants transforms vegetative leaves to cotyledon-like organs, suggesting that these components are also required for the specification of cotyledon identity (Stone *et al.*, 2001; Gazzarrini *et al.*, 2004).



The developmental regulators, *LEC1*, *LEC2* and *FUS3*, are co-ordinately regulated in embryonic and post-embryonic development. In *pickle* (*pkl*) mutants, expression of these genes is up-regulated during post-embryonic development (Rider *et al.*, 2003). The *pkl* mutants were identified based on the mis-expression of embryonic traits in the root (Ogas *et al.*, 1997). The *pkl* phenotype is exaggerated when ABA or a GA biosynthesis inhibitor, uniconazole-P, is applied. The *PKL* gene encodes a chromatin remodelling protein that belongs to CHD3 family (Ogas *et al.*, 1999). These findings suggest that the expression of *LEC1*, *LEC2*, and *FUS3* is repressed epigenetically during post-embryonic development (Eshed *et al.*, 1999). Further evidence demonstrating co-ordinated regulation of *LEC/FUS3/ABI3* expression is provided by ectopic expression experiments. The over-expression of *LEC1* using an inducible promoter caused simultaneous induction of *ABI3* and *FUS3* expression in seedlings (Kagaya *et al.*, 2005). Similarly, short-term induction of *LEC2* leads to ectopic expression of *LEC1*, *FUS3* and *ABI3* (Mendoza *et al.*, 2005). Further research will be needed to clarify the regulatory interaction of these key developmental regulators, which is essential for the understanding of molecular aspects of seed development.

### 11.2.3 Regulators of ABA responses in the seed

The use of genetic approaches has been highly successful in identifying loci involved in ABA responses in the seed. Among the *Arabidopsis abi* (*abscisic acid-insensitive*) mutants, *abi3*, *abi4* and *abi5* show remarkable ABA insensitivity, most specifically in germinating seeds. *abi3* null alleles produce desiccation intolerant seeds, whereas *abi4* and *abi5* seeds display a similar tolerance to wild-type seeds. Orthologues of *ABI3*, *ABI4* and *ABI5* have been identified from various plant species including cereals and crops, indicating that these regulatory mechanisms are common in higher plants.

The *ABI5* and *ABI4* genes encode bZIP and AP2-type transcription factors, respectively (Finkelstein *et al.*, 1998; Finkelstein & Lynch, 2000; Lopez-Molina & Chua, 2000). The *ABI5* protein is capable of binding to G-box-like ABA-responsive elements (ABREs) (Bensmihen *et al.*, 2002; Carles *et al.*, 2002), whereas *ABI4* and maize ortholog *ZmABI4* bind to the coupling element1 (CE1) (Niu *et al.*, 2002; Acevedo-Hernández *et al.*, 2005) that acts cooperatively with ABREs. The contribution of these elements to determining *Arabidopsis* transcriptomes of stored mRNAs was recently shown using microarray analysis (Nakabayashi *et al.*, 2005). Statistical analysis indicated that the G-box-like ABREs are over-represented most significantly in upstream regions of genes whose transcripts accumulate in dry seed. The over-representation of this element is indeed alleviated in the *abi5* dry seeds, indicating that these G-box-like elements act as ABREs *in vivo*. In addition, the RY repeat and CE1 tend to co-exist with ABRE in the genes whose transcripts are highly accumulated in dry seeds. This is consistent with the previous finding that ectopic expression of maize *VP1*, in *Arabidopsis*, causes up-regulation of ABRE-containing genes (Suzuki *et al.*, 2003). Collectively, these results demonstrate that

ABI5, which acts cooperatively with ABI3 and ABI4, is the key determinant for the stored mRNA transcriptome composition in *Arabidopsis* dry seeds.

#### 11.2.4 ABA and GA metabolism genes during seed development

During seed development, the levels of ABA and bioactive GAs are thought to be correlated negatively, as is shown in developing cereal grains (Jacobsen & Chandler, 1987). An increase in seed abortion in the pea (*Pisum sativum*) *lh-2* mutant indicates that GA is essential for embryo growth in this species (Swain *et al.*, 1997). The *lh-2* mutation was recently shown to be a single base substitution in the *ent*-kaurene oxidase gene (Davidson *et al.*, 2004). A prominent role of GAs in embryo growth has also been shown in *Arabidopsis*, where over-expression of a GA-deactivation enzyme gene (pea GA 2-oxidase2) in *Arabidopsis* increases the proportion of seed abortion (Singh *et al.*, 2002). Concomitant with the reduction of bioactive GA levels, ABA levels increase when the maturation phase is initiated. It appears that there are two peaks of ABA accumulation in the mid and late stages during seed development. The first peak of ABA is synthesized in both zygotic and maternal tissues (Karssen *et al.*, 1983). On the other hand, ABA at the second peak is derived from the zygotic tissues. This zygotic-derived ABA is thought to be essential for the induction and maintenance of seed dormancy. In contrast, the maternal-derived ABA is involved in the inhibition of precocious germination and processes of seed maturation in tomato, tobacco and *Arabidopsis* (Koornneef *et al.*, 1989; Groot *et al.*, 1991; Raz *et al.*, 2001; Frey *et al.*, 2004).

In recent years, almost all of the genes necessary for the biosynthesis and deactivation of ABA have been identified in *Arabidopsis* (Chapter 1; Nambara & Marion-Poll, 2005). These genes have provided the opportunity to study the molecular basis of hormone metabolisms during seed development and germination. In the ABA-biosynthetic pathway, the oxidative cleavage of 9-*cis*-epoxycarotenoids catalyzed by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) is an important regulatory step. The *Arabidopsis* genome contains five possible *AtNCED* genes (Iuchi *et al.*, 2001; Tan *et al.*, 2003). Recent work has shown that *AtNCED6* and *AtNCED9* are the major isoforms involved in regulating *Arabidopsis* seed development (Lefebvre *et al.*, 2006). Loss-of-function of these genes lead to a decrease in ABA levels in dry seed, and the double mutant exhibits reduced seed dormancy. On the other hand, ABA 8'-hydroxylation catalyzed by CYP707As is a key regulatory step in the ABA deactivation in various physiological processes. In *Arabidopsis*, four members of CYP707A (CYP707A1 to CYP707A4) are recently shown to encode ABA 8'-hydroxylases (Kushiro *et al.*, 2004; Saito *et al.*, 2004). These genes are expressed differentially during seed development through to germination, suggesting that each CYP707A plays a distinct role in each physiological process. *CYP707A1* and *CYP707A3* are expressed predominantly during seed development, whereas *CYP707A2* is responsible for regulating ABA levels after seed imbibition (Kushiro *et al.*, 2004).

In contrast to the active ABA metabolism in seed development, GA biosynthesis appears to be more active in the early stage of seed development (Kim *et al.*,

2005). An important regulatory step in the production of bioactive GAs is catalyzed by GA 3-oxidases (Yamaguchi & Kamiya, 2001; Hedden *et al.*, 2002). In *Arabidopsis*, there are four GA 3-oxidase genes, *AtGA3ox1* to *AtGA3ox4*. Kim *et al.* (2005) reported that the expression of *AtGA3ox1* and *AtGA3ox4* was induced transiently in early immature seeds. The *AtGA3ox4* expression appears to be localized in outer integuments and the epidermal layer of embryos. Loss-of-function of the *AtGA3ox4* leads to defective seed coat development, which is rescued by application of GA. On the other hand, expression of *AtGA3ox1* and *AtGA3ox2* throughout embryogenesis appears to be restricted to the apical and basal meristem, respectively (Mitchum *et al.*, 2006). *AtGA2ox6* encoding a GA deactivation enzyme, GA 2-oxidase, is shown to be highly expressed in the embryo during early embryogenesis as well as seed germination (Wang *et al.*, 2004). It has been shown that expression of the *AtGA2ox6* is directly regulated by a MADS box transcription factor AGAMOUS like 15, a regulator of embryo development (Wang *et al.*, 2004). The loss-of-function of the *AtGA2ox6* leads to an enhanced germination capacity, indicating that *AtGA2ox6* is a regulatory isoform of GA 2-oxidase during seed germination (Wang *et al.*, 2004).

#### 11.2.5 Regulation of balancing ABA and GA levels during seed development

Recent reports have demonstrated that ABA and GA biosynthesis during seed development are regulated by common factors in an opposite manner. The *FUS3* gene has been shown to regulate the levels of both ABA and GA during seed development. Gazzarrini *et al.* (2004) found that the ectopic expression of *FUS3*, driven by an epidermis-specific *AtML1* promoter, transforms vegetative organs to those that are embryo-like, by a non-cell-autonomous process. The *FUS3*-induced transformation is alleviated by the introduction of an ABA-deficient mutation, *aba2*, or the application of bioactive GA. Moreover, the transient induction of *FUS3* resulted in ABA accumulation and repression of the GA-biosynthetic genes, *AtGA20ox1* and *AtGA3ox1*. These findings indicate that *FUS3* acts as a positive regulator of ABA levels and a negative regulator of GA levels during seed development. Consistent with these roles for *FUS3*, the *fus3-3* mutant exhibits delayed ABA accumulation during the seed maturation stage. Importantly, *FUS3* protein levels appear to be regulated by ABA and GA in a positive and negative way, respectively. These regulatory loops support a role for *FUS3* as a metabolic switch that regulates ABA and GA levels (Fig. 11.2b).

A better understanding of the mechanisms by which these heterochronic genes regulate GA biosynthesis has come from the finding that *FUS3* directly regulates the expression of GA-biosynthetic genes. Initially, Curaba *et al.* (2004) reported that bioactive GAs levels increased in the immature seeds of the *fus3* and *lec2* mutants (Curaba *et al.*, 2004). The immature embryos of these mutants were subsequently found to have higher levels of *AtGA20ox1* and *AtGA3ox2* transcripts than those of wild-type plants. In these mutants, the mis-expression of *AtGA3ox2* was observed in the epidermis of embryo axis and vascular tissues, which coincide

with the localization of the *FUS3* expression (Tsuchiya *et al.*, 2004). Furthermore, *FUS3*, but not *LEC2*, interacts physically with the RY repeats located in the *AtGA3ox2* promoter, presumably to repress *AtGA3ox2* expression (Curaba *et al.*, 2004). It is worth noting that the *fus3* and *lec2* mutants differentially over-accumulate the bioactive GAs, GA<sub>1</sub> and GA<sub>4</sub>, suggesting that *FUS3* and *LEC2* regulate GA biosynthesis through distinct mechanisms.

### 11.2.6 Regulation of ABA and GA action during seed development

Hormone sensitivity is regulated differentially in a particular developmental and physiological process. The molecular interaction between ABA signaling components and developmental regulators was initially demonstrated in rice. Hobo *et al.* (1999) screened for protein interactors with OsVP1, the rice VP1/ABI3 ortholog. One of the interactors that specifically binds to the truncated OsVP1, harbouring B1 and B2 domains, was TRAB1, a bZIP-type transcription factor. TRAB1 was shown to bind to ABRE and transactivate ABRE-mediated transcription synergistically with OsVP1. Accordingly, the developmental regulator VP1/ABI3 controls ABRE-mediated transcription in the seed *via* direct interaction with the ABRE-binding protein TRAB1. In *Arabidopsis*, ABI3 and ABI5 were shown to interact in yeast two-hybrid assay (Fig. 11.2c) (Nakamura *et al.*, 2001). The B1 domain of ABI3 appears to be responsible for the interaction with ABI5. In contrast, other B3 proteins, *FUS3* and *LEC2*, do not contain the B1 domain, and it is unlikely that these interact with ABI5. This might reflect the phenotypic difference between *abi3* and *fus3/lec2* mutants in ABA sensitivity.

In contrast to the role of ABA, much less is known concerning GA signaling in seed development. Nevertheless, the antagonistic actions of ABA and GA, that are well-characterized in germinating seeds, are also apparent during seed development. The maize ABA-insensitive *vp1* mutants show defects in seed reserve accumulation, acquisition of desiccation tolerance and induction of seed dormancy (McCarty *et al.*, 1989). Concomitant with such defects in seed maturation processes, a set of processes that are normally observed upon wild-type germination are precociously expressed during *vp1* seed development, including precocious induction of GA-responsive  $\alpha$ -amylase gene expression (Hoecker *et al.*, 1995). Transient introduction of VP1 into immature *vp1* seeds can repress the precocious induction of  $\alpha$ -amylase gene, suggesting that GA action is suppressed by VP1 during seed development in maize (Hoecker *et al.*, 1995).

## 11.3 Hormonal control of seed germination and post-germinative growth

### 11.3.1 Regulation of GA levels in imbibed seeds

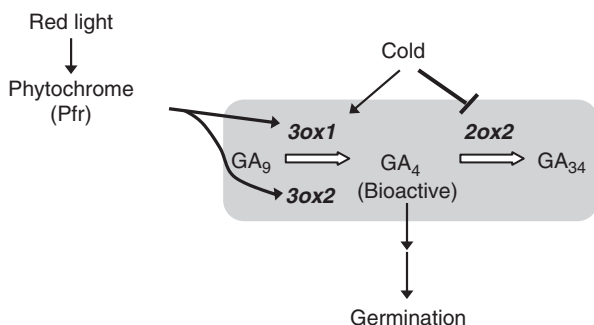
Non-dormant seeds germinate upon water uptake when they are exposed to favourable light, temperature and nutrient conditions. There is accumulating evidence to indicate

that the regulation of seed germination by environmental signals is, in part, mediated by hormones, particularly by GAs. Here, we summarize our current understanding on light- and temperature-regulation of GA biosynthesis and response pathways.

### 11.3.1.1 Light-regulation of GA biosynthesis

Seed germination is light-dependent and under phytochrome control in some small-seeded plants, such as lettuce, tomato and *Arabidopsis* (reviewed in Shinomura, 1997). Light-regulated seed germination was first recognized in dark-imbibed lettuce seeds, where red (R) light induces, but far-red (FR) light reversibly inhibits germination (Borthwick *et al.*, 1952). In dark-imbibed lettuce seeds, endogenous GA<sub>1</sub> (the major bioactive GA in lettuce seeds), but not its immediate precursor GA<sub>20</sub>, increases in abundance after R-light treatment (Toyomasu *et al.*, 1993). The effect of R-light on the GA<sub>1</sub> level is cancelled when FR is irradiated following the R-light treatment, suggesting the involvement of phytochrome. Since the precursor GA<sub>20</sub> accumulates at a high level (about 100 times that of GA<sub>1</sub>) in dark-imbibed lettuce seeds, the conversion of GA<sub>20</sub> to GA<sub>1</sub> is likely to be the limiting step for the production of GA<sub>1</sub>. Consistent with these observations, the level of *Ls3h* transcript, encoding a GA 3-oxidase that catalyzes the conversion of GA<sub>20</sub> to GA<sub>1</sub>, increases in response to R-light (Toyomasu *et al.*, 1998). In contrast, transcript levels of two GA 20-oxidases are induced by imbibition alone regardless of the light regime. A slight reduction in the level of *LsGA2ox2* transcript by R-light treatment suggests that GA deactivation might also be regulated by phytochrome in lettuce seeds (Nakaminami *et al.*, 2003).

In *Arabidopsis*, five genes encoding phytochromes, *PHYA* to *PHYE*, have been identified (Sharrock & Quail, 1989). Among them, *PHYB* is the major family member that is stored in seeds at maturity, and is responsible for the typical photoreversible response shortly after the start of imbibition (Shinomura *et al.*, 1994; Shinomura *et al.*, 1996). In dark-imbibed *Arabidopsis* seeds, transcript accumulation of the *AtGA3ox1* and *AtGA3ox2* genes, both of which encode GA 3-oxidase, is elevated by a brief R pulse, and the effect of R-light is reversed by FR-light (Fig. 11.3; Yamaguchi *et al.*, 1998). In the *phyB* mutant, *AtGA3ox2* expression is not increased by R-light, indicating a primary role of *PhyB* in mediating the R-light induction of *AtGA3ox2*. It is still unclear which phytochrome species is responsible for R-induced *AtGA3ox1* expression (Yamaguchi *et al.*, 1998). In *Arabidopsis*, GA 3-oxidases are encoded by at least four genes (Hedden *et al.*, 2002). The non-germinating phenotype of the digenic *atga3ox1/atga3ox2* mutant shows that the phytochrome-regulated *AtGA3ox1* and *AtGA3ox2* genes encode major GA 3-oxidases necessary for seed germination (Mitchum *et al.*, 2006). So far, phytochrome-regulation of GA biosynthesis has been studied only in the classical photoreversible response to R- and FR-light pulses given immediately after imbibition, which is mainly mediated by *PHYB* in *Arabidopsis*. However, additional phytochromes are in fact involved in sensing a variety of light regimes (wavelength, duration and intensity) in imbibed *Arabidopsis* seeds. For example, *PHYA* accumulates in the



**Figure 11.3** Environmental regulation of GA biosynthesis and deactivation in imbibed *Arabidopsis* seeds. White thick arrows indicate metabolic conversions. Positive-regulation is shown by black arrows. T-bar depicts negative-regulation. *3ox1* (*AtGA3ox1*) and *3ox2* (*AtGA3ox2*) encode GA 3-oxidases. *2ox2* (*AtGA2ox2*) encodes a GA 2-oxidase.

seed during a long period of dark-imbibition (e.g. 48 h), and plays a role in the irreversible response to irradiation with extremely low fluence light in a wide range of wavelength (very low fluence response) (Shinomura *et al.*, 1996). In addition, both PHYA and PHYE are required for germination in continuous FR-light (Hennig *et al.*, 2002). It is therefore likely that multiple phytochromes play distinct and overlapping roles in sensing light in natural conditions. It will be valuable to examine regulation of GA biosynthesis by such phytochrome species in defined light conditions to better understand how light signals control seed germination through changes in hormone levels. Recently, a phytochrome-interacting basic helix–loop–helix protein, PIL5, has been shown to function as a negative regulator of seed germination (Oh *et al.*, 2004). Investigations into the relationships between such light signaling components and GA biosynthesis genes may help to uncover the molecular mechanisms for phytochrome-regulation of GA biosynthesis.

#### 11.3.1.2 Temperature-regulation of GA biosynthesis

Temperature is another important environmental factor that controls seed germination. Exposure of imbibed seeds to cold temperature (stratification) accelerates the release from seed dormancy in many plant species (Bewley & Black, 1982). Due to its positive effect on seed germination, cold treatment (pre-incubation at cold temperature in the dark) has been studied as a potential regulator of GA biosynthesis in several plant species. In *Pyrus malus* (apple), *Corylus avellana* (hazel) and *Arabidopsis*, cold treatment improved the ability of seeds to synthesize bioactive GAs during seed germination (Ross & Bradbeer, 1971; Sínska *et al.*, 1973; Williams *et al.*, 1974; Derkx *et al.*, 1994). More recently, the effect of cold temperature on the GA biosynthesis pathway has been investigated in detail in dark-imbibed *Arabidopsis* seeds (Yamauchi *et al.*, 2004). During dark-imbibition at 4°C (after an FR-light pulse to inactivate pre-existing phytochrome), the level of *AtGA3ox1* mRNA increases to a high level, while it remains low during dark-imbibition at



22°C. In contrast, the level of *AtGA2ox2* transcript is much higher at 22°C than at 4°C in seeds imbibed in the dark. These results suggest that the synthesis of bioactive GAs is activated, while GA deactivation is suppressed, by cold temperature (Fig. 11.3). In agreement with these notions, the levels of GA<sub>4</sub> and GA<sub>1</sub> in dark-imbibed seeds are elevated at 4°C compared to those at 22°C (Yamauchi *et al.*, 2004). Among four *AtGA3ox* genes in *Arabidopsis*, only *AtGA3ox1* is induced by cold temperature in dark-imbibed seeds (Yamauchi *et al.*, 2004). The increase in the amounts of bioactive GAs, cold-induction of GA-up-regulated genes and cold-stimulated seed germination did not occur in the *atga3ox1* mutant, demonstrating that *AtGA3ox1* is required for mediating the temperature signal.

### 11.3.1.3 GA response components in germinating seeds

In recent years, several GA signaling components have been identified by genetic approaches utilizing mutants with increased or decreased GA response, and their roles in various aspects of plant growth and development are being studied extensively (for reviews, see, Olszewski *et al.*, 2002; Gomi & Matsuoka, 2003; Itoh *et al.*, 2003; Sun & Gubler, 2004; Thomas & Sun, 2004; Fleet & Sun, 2005). Here, we cover the GA response pathway with focus on its relation to environmental regulation of seed germination in *Arabidopsis*.

Light modulates the apparent sensitivity of seeds to exogenous GA during germination in multiple plant species (Hilhorst & Karssen, 1988; Derkx & Karssen, 1993; Yang *et al.*, 1995; Li *et al.*, 2005). In *Arabidopsis*, GA sensitivity is photoreversibly altered by R and FR-light, suggesting the involvement of phytochrome (Yang *et al.*, 1995). DELLA proteins form a subfamily in the GRAS family of putative transcription factors, and act as negative regulators in the GA response pathway. GA de-represses its signaling pathway by inducing proteolysis of the DELLA proteins through the ubiquitin-26S proteasome pathway (Itoh *et al.*, 2003; Sun & Gubler, 2004; Thomas & Sun, 2004). Among five *DELLA* genes (*RGA*, *GAI*, *RGL1*, *RGL2* and *RGL3*) in *Arabidopsis*, *RGL2* has been shown to encode the major negative regulator during seed germination; loss-of-function *rgl2* mutations, but none of the *rga*, *gai*, *rgl1* and *rgl3* single mutations, are able to suppress the non-germinating phenotype of the GA-deficient *gal-3* mutant, although other members are also expressed in germinating seeds (Lee *et al.*, 2002; Tyler *et al.*, 2004; Cao *et al.*, 2005). Interestingly, *rga/rgl1/rgl2* and *gai/rgl1/rgl2* triple knockout mutations confer GA-dependent germination in the light, but not in the dark, whereas a *rga/gai/rgl2* triple mutation allows germination of *gal-3* seeds regardless of the light condition (Cao *et al.*, 2005). These results imply that *RGA* and *GAI* may be involved in alterations of the GA response pathway by light in imbibed seeds. It is also intriguing that *RGA* is present at a higher level than *RGL2* in imbibed seeds, in spite of the genetic evidence that *RGL2* plays a more prominent role than does *RGA* in suppressing seed germination (Tyler *et al.*, 2004). Detailed time-course analysis indicates that down-regulation of *RGL2* mRNA accumulation is not essential for seed germination, as it occurs at a post-germinative phase (Bassel *et al.*, 2004). Altogether, current experimental evidence suggests the importance of investigations into the role of DELLA



genes in seed germination at the protein level, including the cell-type specificity in seeds (see below) and regulation by environmental signals.

Cold treatment decreases the amount of exogenous GA necessary for inducing germination of GA-deficient mutant seeds in *Arabidopsis* (Derks & Karssen, 1993; Derks *et al.*, 1994). Therefore, a change in sensitivity to GA might also play a role in the stimulation of seed germination by cold temperature. At present, molecular mechanisms underlying this phenomenon are unknown.

### 11.3.2 Regulation of ABA levels in imbibed seeds

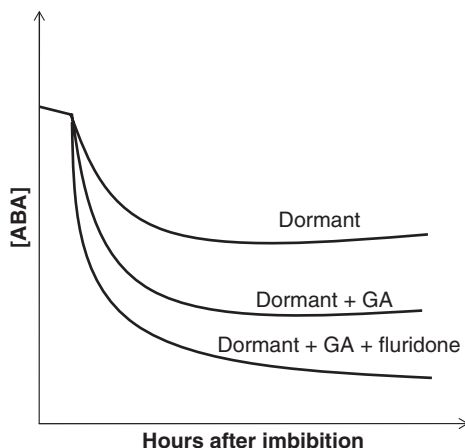
#### 11.3.2.1 De novo ABA biosynthesis and deactivation are involved in regulation of ABA levels

In general, mature dry seed contains a significant amount of ABA. The ABA level decreases rapidly after seed imbibition, and is then maintained at a particular level that is determined by environmental and developmental factors. There have been many reports using various plant species, demonstrating the release of seed dormancy following a pretreatment with an inhibitor of ABA biosynthesis, fluridone (Toyomasu *et al.*, 1994; Yoshioka *et al.*, 1998; Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004). This implies that after seed imbibition, the ABA level is determined by a balance of *de novo* biosynthesis and deactivation, but not by deactivation alone.

#### 11.3.2.2 Light, high temperature, and GA regulation of ABA metabolism

ABA levels have been analysed during photoreversible seed germination in lettuce. In lettuce (*Lactuca sativa* L. cv Grand Rapids), seed germination is strictly regulated by light and temperature. In dark-imbibed lettuce seeds, germination is prevented by a FR-light pulse irradiation, whereas a subsequent R-light pulse treatment or GA application acts to promote it. The FR-treated seed maintained a high ABA level and the R treatment or GA application elicit a rapid reduction in ABA levels, which occurs prior to seed germination (Toyomasu *et al.*, 1994). Lettuce seed germination is also blocked when imbibition occurs at a high temperature (33°C) in darkness. The effect of this thermoinhibition is alleviated partially when fluridone and GA are co-applied exogenously (Gonai *et al.*, 2004). The high-temperature treatment results in the imbibed seeds accumulating higher levels of ABA compared with those incubated at room temperature. Treatment with fluridone or GA alone leads to a decrease in ABA levels prior to germination, and an additive effect of co-application of these chemicals to decrease the ABA levels was observed prior to germination (Fig. 11.4). Since the GA treatment increases the levels of phaseic acid (PA) and dihydrophaseic acid (DPA), compared with those of ABA conjugates, one of the effects of GA action in this response is likely to enhance the rate of ABA 8'-hydroxylation.

In *Arabidopsis*, changes in endogenous ABA levels during dormancy release has been studied in the Cape Verde Islands (Cvi) ecotype, which exhibits prominent seed dormancy (Ali-Rachedi *et al.*, 2004). The dormancy of Cvi seeds is released by after-ripening, stratification, and by treatment with nitrate or fluridone. GA treatment has little effect on breaking dormancy in this accession. Imbibed



**Figure 11.4** Changes in endogenous ABA levels after seed imbibition. ABA levels decrease rapidly after seed imbibition. This reduction in ABA levels is completed within 6–12 h after imbibition depending on plant species. Dormant seed maintains a higher ABA level compared with that of non-dormant imbibed seed. GA and fluridone treatments reduce ABA levels and permit seed germination.

dormant seeds maintain higher ABA levels compared to non-dormant seeds especially after 3 days of imbibition. These findings indicate that high levels of ABA are important for maintenance of seed dormancy after imbibition. The recent identification of genes encoding ABA 8'-hydroxylases reveals that CYP707A2 is responsible for the rapid decrease in ABA levels after seed imbibition (Kushiro *et al.*, 2004). The CYP707A2 expression is induced after seed imbibition and loss-of-function of this gene leads to hyperdormancy of the seeds.

### 11.3.3 Sites of GA biosynthesis and response in imbibed seeds

The tomato GA-deficient mutant, *gib-1* requires exogenous GA treatment for germination to occur. However, removal of the endosperm and testa layers around the radicle tip will bypass this requirement (Groot & Karssen, 1987). Likewise, when the structure covering the *Arabidopsis* embryo (aleurone and seed coat) is mechanically removed, the GA-deficient *gal-3* embryos can grow into dwarfed seedlings (Silverstone *et al.*, 1997; Telfer *et al.*, 1997). Therefore, GA has been thought to play a role in overcoming the physical constraint of the surrounding structure, endosperm or seed coat or both, by inducing their weakening (Bewley, 1997b).

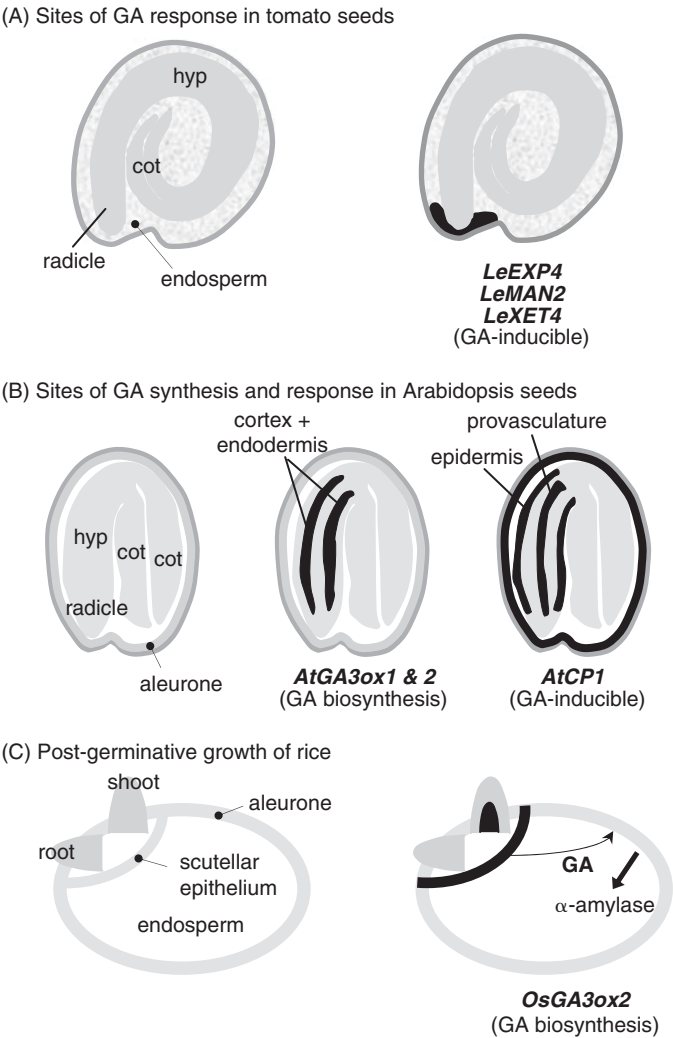
Weakening of the endosperm and testa during germination was estimated by measuring the puncture force needed to break through these layers at the micropylar region (where the radicle protrudes) in tomato seeds (Groot & Karssen, 1987). In wild-type seeds, the puncture force decreases before radicle emergence. However, in *gib-1* seeds a reduction in the necessary puncture force is only observed in the presence of exogenous GA. When isolated endosperm and testa (embryo-less half seeds) of the

*gib-1* mutant are incubated with isolated wild-type embryos, the puncture force decreases significantly (Groot & Karssen, 1987). These results suggest that the weakening of the endosperm and testa is dependent on GA produced in the embryos. Although actual movement of GA from the embryo to the endosperm has not been demonstrated, this hypothesis is consistent with the expression of GA biosynthesis genes in the embryos of germinating *Arabidopsis* seeds (see below).

There is evidence to suggest that GA weakens the endosperm and testa by increasing the levels of cell wall modifying enzymes. This is illustrated in tomato seeds where GA-dependent breakdown of the endosperm cell wall results in the release of mannose (Groot *et al.*, 1988), and endo- $\beta$ -mannanase activity is detected exclusively in the micropylar endosperm prior to germination (Nonogaki & Morohashi, 1996). In agreement with this observation, *LeMAN2* mRNA, encoding an endo- $\beta$ -mannanase, accumulates specifically in the micropylar endosperm before radicle protrusion (Fig. 11.5a; Nonogaki *et al.*, 2000). Expansins are involved in cell wall extension, possibly by disrupting hydrogen bonding between the cell wall components (Cosgrove, 1998). The *LeEXP4* gene, encoding an expansin, is specifically expressed in the micropylar endosperm cap prior to radicle protrusion in a GA-dependent manner (Fig. 11.5a; Chen & Bradford, 2000). A gene encoding xyloglucan endotransglycosylase, *LeXET4*, exhibits a similar GA-dependent, tissue-specific expression pattern (Fig. 11.5a; Chen *et al.*, 2002). Tissue-specific expression of these GA-up-regulated genes identify the micropylar endosperm cap as a site of GA action, and supports the role of GA in facilitating the weakening of the mechanical barrier imposed by the tissues surrounding the embryo.

Sites of GA biosynthesis in seeds have been studied in *Arabidopsis* by analysing the expression of GA biosynthesis genes at cellular resolution. In light-imbibed *Arabidopsis* seeds, *AtGA3ox1* and *AtGA3ox2* transcripts are localized to the cortex and endodermis of embryonic axes, suggesting that these are the major sites for the synthesis of bioactive GAs (Fig. 11.5b; Yamaguchi *et al.*, 2001). GeneChip microarray analysis has identified a number of transcripts, the abundance of which is regulated in response to exogenous GA<sub>4</sub> in imbibed non-germinating *gal-3* mutant seeds (Ogawa *et al.*, 2003). Interestingly, expression of some GA-inducible genes is not restricted to the predicted site of bioactive GA synthesis in germinating wild-type seeds. For example, the GA-up-regulated *AtCPI* transcript (encoding a cysteine proteinase) is localized to the epidermis and provasculture of embryonic axes and the aleurone (endosperm) layer, whereas transcripts for the major GA 3-oxidases are mainly detected in the cortical and endodermal cell layers (Fig. 11.5b). These observations suggest that GA itself, or GA signal(s), might be transmitted across different cell layers in germinating *Arabidopsis* seeds (Ogawa *et al.*, 2003).

In germinating cereal grains, aleurone cells play a role in assisting the mobilization of nutrients stored in the seed by secreting hydrolases that degrade starch, protein and lipid. In imbibed *Hordeum vulgare* (barley) grains, the major increase in GA content occurs after the start of radicle emergence (Jacobsen *et al.*, 2002), and a necessity for GA for germination of cereal grains has been elusive, unlike the cases with *Arabidopsis* and tomato. However, it is clear that the synthesis of bioactive



**Figure 11.5** Sites of GA biosynthesis and response in germinating seeds. (a) Sites of GA response in tomato seeds. Left; Schematic drawing of a longitudinal section through a tomato seed. Cot, cotyledon; Hyp, hypocotyl. Right; Localization of GA-inducible *LeMAN2*, *LeEXP4* and *LeXET4* transcripts in the micropylar region of the endosperm (depicted in black). (b) Sites of GA biosynthesis and response in Arabidopsis seeds. Left; Schematic drawing of a longitudinal section of an Arabidopsis seed. Cot, cotyledon; Hyp, hypocotyl. Middle; Localization of the *AtGA3ox1* and *AtGA3ox2* transcripts in the cortical and endodermal cells of embryonic axis. Right; Cell type-specific expression of a GA-upregulated gene, *AtCPI* (depicted in black). (c) Sites of GA biosynthesis during post-germinative growth of rice. Left; Schematic illustration of a germinated rice grain. Right; Localization of the *OsGA3ox2* transcript in the scutellar epithelium and the apical part of shoots (depicted in black). The thin arrow indicates the movement of GA from the epithelium to the aleurone. The thick arrow denotes the secretion of  $\alpha$ -amylase from the aleurone to the endosperm.

GA is required for the production of hydrolases in the aleurone in imbibed cereal grains (Appleford & Lenton, 1997). Measurements of *ent*-kaurene accumulation in imbibed wheat grains treated with paclobutrazol (an inhibitor for *ent*-kaurene metabolism) indicate that the scutellum and the shoot are the major sites of *de novo* GA biosynthesis (Appleford & Lenton, 1997). In rice, GA 3-oxidase is encoded by two genes, *OsGA3ox1* and *OsGA3ox2* (Itoh *et al.*, 2001). In germinating rice seeds, *OsGA3ox2*, plays a dominant role in  $\alpha$ -amylase gene expression in the aleurone (Kaneko *et al.*, 2002). The *OsGA3ox2* transcript accumulates specifically in the scutellar epithelium and the apical region of growing shoots (Fig. 11.5c). The synthesis of bioactive GAs in the epithelium is important for  $\alpha$ -amylase induction in the aleurone, because an embryonic mutant defective in shoot formation, but not a mutant defective in epithelium development, is able to induce  $\alpha$ -amylase gene expression. Taken together, *OsGA3ox2* expressed in the epithelial cells is likely to be responsible for *de novo* GA synthesis necessary for  $\alpha$ -amylase induction in the aleurone (Kaneko *et al.*, 2002).

#### 11.3.4 GA and ABA action in the cereal aleurone

Over the past few decades, the cereal aleurone of germinating grains has been a valuable system to study GA and ABA signaling using well-characterized hormone-responsive genes as molecular markers. Pharmacological studies using hormone-responding protoplasts and the use of effector and reporter constructs in transient gene expression analysis have contributed to the identification of components in the ABA and GA signaling pathways in cereal aleurone. Recently, genes originally identified by molecular genetic analysis using GA/ABA response mutants have proven to be common signaling components in the aleurone system as well. Although GA and ABA antagonism has long been studied as an important factor controlling seed dormancy and germination, our knowledge is still limited on how molecular interactions between GA and ABA response pathways are achieved. Cereal aleurone serves as an advantageous system in which to pinpoint nexus(es) of the signaling pathways, as it consists of a single cell type. Several review articles have described the GA and ABA signaling pathways in cereal aleurone in detail (Lovegrove & Hooley, 2000; Olszewski *et al.*, 2002; Sun & Gubler, 2004). In this section, we start with our current understanding on the hormone perception in cereal aleurone cells, and then describe some major components in the GA and ABA signaling pathways to highlight briefly how these two hormones interact to regulate gene expression.

##### 11.3.4.1 GA and ABA perception

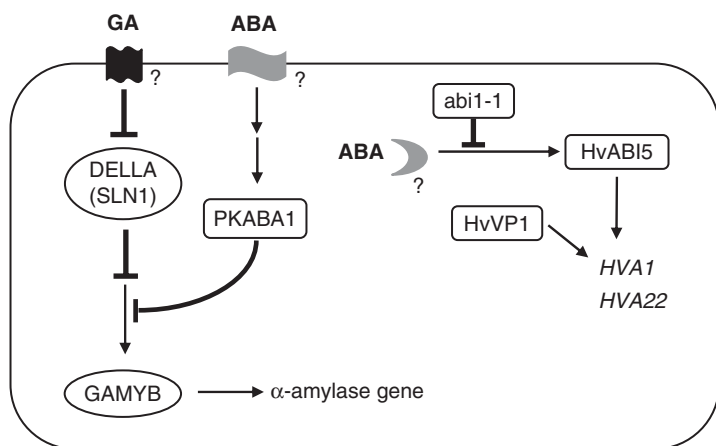
There are two lines of circumstantial evidence that suggest that GA is perceived at the outer surface of the plasma membrane in the cereal aleurone. First, Sepharose beads covalently bound with GA<sub>4</sub> is able to induce  $\alpha$ -amylase gene expression in aleurone protoplasts of wild oat, despite the fact that they are not permeable through the plasma membrane (Hooley *et al.*, 1991). Second, GA does not stimulate

$\alpha$ -amylase gene expression when it is micro-injected into barley aleurone cells, unlike the case with exogenous GA in the incubation media (Gilroy & Jones, 1994). Recently, a soluble GA receptor has been identified through characterization of a recessive GA-insensitive dwarf mutant of rice, *gid1* (Ueguchi-Tanaka *et al.*, 2005; see Chapter 6). In germinating *gid1* mutant seeds,  $\alpha$ -amylase activity is undetectable even at a high concentration of exogenous GA. This observation indicates that *GID1* is required for GA-induced  $\alpha$ -amylase expression in the aleurone, in which GA is thought to be recognized at the plasma membrane. Therefore, a role of *GID1* in GA perception in cereal aleurone cells remains to be investigated further.

Microinjection of ABA into GA-treated barley aleurone protoplasts is not effective in inhibiting  $\alpha$ -amylase gene expression (Gilroy & Jones, 1994), while ABA in the incubation media is. Therefore, the ABA inhibition of GA action in the aleurone system is likely to be mediated by ABA perception at the plasma membrane. Interestingly, induction of an ABA-up-regulated *Em* promoter occurs when ABA is micro-injected into aleurone protoplasts, suggesting that this ABA response is mediated by ABA perception inside the protoplast (Gilroy, 1996). Thus, cereal aleurone cells appear to have two sites of ABA perception (Fig. 11.6), although the identity of these ABA receptors is currently unknown.

#### 11.3.4.2 Crosstalk between GA and ABA action

An MYB transcription factor, HvGAMYB, is a GA-up-regulated transcriptional activator of  $\alpha$ -amylase gene expression in barley aleurone cells (Gubler *et al.*, 1995). HvGAMYB is able to transactivate  $\alpha$ -amylase and other GA-responsive promoters in the absence of exogenous GA (Gubler *et al.*, 1995). HvGAMYB, is not only sufficient, but also necessary for the GA induction of  $\alpha$ -amylase in barley aleurone, as proved by transiently expressed RNA-interference in protoplast assays



**Figure 11.6** Crosstalk between ABA and GA action in barley aleurone. Arrows indicate positive-regulation. T-bar depicts negative-regulation.

(Zentella *et al.*, 2002). GA causes a 2-fold increase in the rate of *HvGAMYB* transcription, and this effect can be partly blocked by ABA (Fig. 10.7; Gubler *et al.*, 2002; Sun & Gubler, 2004). A DELLA protein, SLN1, which is the product of the *Slender1* locus of barley, is necessary for repression of *HvGAMYB* in barley aleurone cells (Gubler *et al.*, 2002), which indicates that SLN1 acts upstream of *HvGAMYB*. GA treatment rapidly decreases SLN1 protein levels before the increase in *HvGAMYB* levels. GA-induced SLN1 degradation is not blocked by ABA (Gubler *et al.*, 2002), indicating that ABA acts downstream of SLN1 to block GA signaling (Fig. 11.6). Transient over-expression of an ABA-inducible Ser/Thr protein kinase, PKABA1, represses the GA induction of  $\alpha$ -amylase and *HvGAMYB* expression, but not *GAMYB*-transactivated  $\alpha$ -amylase gene expression (Gomez-Cadenas *et al.*, 2001). Constitutive *GAMYB* and  $\alpha$ -amylase expression in *sln1* aleurone cells is also repressed by PKABA1 (Gomez-Cadenas *et al.*, 2001). It is therefore likely that PKABA1 mediates ABA suppression of  $\alpha$ -amylase expression at a point that is upstream of the formation of *GAMYB* and downstream of the site of SLN1 action in the GA response pathway (Fig. 11.6). However, suppression of *PKABA1* by RNA-interference does not inhibit the negative effect of ABA on  $\alpha$ -amylase gene expression, suggesting that a PKABA1-independent pathway may also exist (Zentella *et al.*, 2002).

Exogenous ABA induces expression of the *HVA1* and *HVA22* genes, both of which are normally expressed at high levels during late embryogenesis, in the aleurone of germinating barley grains. The ABA induction of *HVA1* and *HVA22* gene expression is inhibited by a mutated version of protein phosphatase 2C, encoded by the dominant *abi1-1* mutant gene that blocks ABA responses in *Arabidopsis* (Shen *et al.*, 2001). However, the *abi1-1* gene product (*abi1-1*) does not affect the ABA suppression of GA-inducible  $\alpha$ -amylase gene expression. Interestingly, PKABA1, which suppresses GA-inducible  $\alpha$ -amylase genes, has little effect on the ABA induction of *HVA1* and *HVA22* genes (Shen *et al.*, 2001). Therefore, it appears that there are two separate ABA signaling pathways; one inhibited by *abi1-1* and the other regulated by PKABA1 (Fig. 11.6). A bZIP transcription factor, HvABI5, recognizes the ABA response promoter complexes (ABRC) of the *HVA1* and *HVA22* genes, and is capable of trans-activating ABRC-reporter genes in barley aleurone cells in the presence of HvVP1 (Casaretto & Ho, 2003). Activation of the *HVA1* and *HVA22* genes by HvABI5/HvVP1 is not inhibited by *abi1* (Fig. 11.6). Therefore, HvABI5 and HvVP1 are necessary for the ABA-up-regulation of *HVA1* and *HVA22* genes, but are dispensable for the ABA suppression of GA-inducible  $\alpha$ -amylase gene expression (Casaretto & Ho, 2003).

### 11.3.5 Other hormones: actions of ethylene and brassinosteroids during seed germination

#### 11.3.5.1 Ethylene

Ethylene is known to be a positive regulator for seed germination in many species (Kepczynski & Kepczynska, 1997). Ethylene can induce germination of *Arabidopsis*



*gal* mutant in the light in the absence of exogenous GA. However, this is not the case for the tomato *gib-1* mutant (Karrsen *et al.*, 1989). On the other hand, ethylene antagonizes ABA in *Arabidopsis* seed germination (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). Beaudoin *et al.* (2000) reported that genetic screening of the enhancer and suppressor of ABA-insensitive germination of the *abil-1* mutant were allelic to *ctr1* and *ein2*, respectively. Ghassemian *et al.* (2000) also found that ABA-hypersensitive *era3* mutants were *ein2* alleles. The ethylene signaling components, CTR1 and EIN2, are negative and positive regulators, respectively (Straeten 2006). The *etr1* mutants that are defective in an ethylene receptor show similar phenotypes, indicating that ethylene and ABA signaling pathways interact antagonistically, rather than CTR1 and EIN2 play a specific role in the crosstalk. Ethylene-insensitive mutants show hyper seed dormancy, which is canceled by stratification or by after-ripening (Ghassemian *et al.*, 2000). This suggests that ethylene acts independently from stratification-induced events during germination. It is also noteworthy that ethylene-insensitive mutants of *Arabidopsis* display ABA-insensitive root growth, suggesting that the mode (or component) of crosstalk between ethylene and ABA signaling pathways is tissue specific.

Crosstalk between ethylene and ABA during germination is not restricted to signaling, but is also evident with respect to ABA metabolism. Chiwocha *et al.* (2005) quantified hormone levels in dry and imbibed *etr1* seeds. The *etr1-2* dry seed contained approximately 10-fold higher ABA levels relative to the wild type. After imbibition, the *etr1-2* seed maintained 2- to 3-fold higher ABA levels compared with the wild type. In the *etr1-2* imbibed seed, levels of dihydrophaseic acid (a catabolite from ABA 8'-hydroxylation), 7'-hydroxy ABA, and ABA glucose ester were lower than those in the wild type, suggesting that ABA deactivation is down-regulated in the *etr1-2* mutant. In addition, the most significantly down-regulated catabolite in the *etr1* mutant was ABA glucose ester. Based on this observation, the authors claimed that ABA glucosylation plays an important role in ethylene-mediated regulation of ABA metabolism.

#### 11.3.5.2 *Brassinosteroids*

Brassinolide (BL) is also known to positively regulate seed germination in several plant species. In *Arabidopsis*, application of BL partially rescues germination of the non-germinating GA-deficient mutants (Steber & McCourt, 2001). BL treatment also alleviates partially ABA-mediated inhibition of *Arabidopsis* seed germination. In addition, ABA-hypersensitive germination was evident in the BL deficient and insensitive mutants, *det2* and *brl1*, respectively. Tobacco seed germination is also accelerated by application of BL (Leubner-Metzger, 2001). The effect of BL application is distinct from that elicited by GA application. Exogenous BL accelerates endosperm rupture in light-imbibed seed, which is not observed after GA treatment. On the other hand, BL fails to release dark-imbibed photodormancy nor induce endosperm-specific class I 1,3- $\beta$ -glucanase activity that is stimulated by GA application. Based on the differences, Leubner-Metzger (2001) proposed that BL promotes tobacco seed germination through enhancing growth potential of the

embryo, which is distinct from the dual functions of GA on seed germination of enhancing embryonic growth potential and weakening the micropylar endosperm. The BL action on seed germination is also suggested to act in a GA-independent manner in *Arabidopsis*, although both of BR and GA actions require heterotrimeric G-proteins (Chen *et al.*, 2004).

## 11.4 Conclusions and perspectives

In this chapter, we have highlighted recent advances in our understanding of hormonal regulation of seed dormancy and germination. Identification of genes in the biosynthesis and deactivation pathways of ABA and GA are allowing researchers to study regulation of the concentrations of these two hormones in seeds at the cellular resolution, in relation to specific developmental phases and under defined environmental conditions (see Chapters 1 and 6). Evidence has been provided that well-characterized developmental regulators, such as FUS3 and LEC2, play a (direct) role in modulating hormone levels in developing seeds (Curaba *et al.*, 2004; Gazzarrini *et al.*, 2004). Identifications of additional key regulators of ABA and GA levels will increase our knowledge on how cellular concentrations of antagonistic hormones are balanced during seed development and germination. The molecular basis of crosstalk between ABA and GA actions is beginning to be uncovered in the cereal aleurone, and this system will continue to be useful to dissect the ABA and GA antagonism at the molecular level and to place additional components in respective hormone signaling pathways (Olszewski *et al.*, 2002; Sun & Gubler, 2004). Applications of the newly available large-scale transcriptome and proteome technologies to seed biology (Ogawa *et al.*, 2003; Rajjou *et al.*, 2004; Nakabayashi *et al.*, 2005) will be powerful aids in developing our understanding of the exact roles of each hormone in seed dormancy and germination. Such genome-wide analyses in combination with the use of molecular genetic tools will allow us to reveal unidentified molecular links among different hormones as well as connections between a hormone and a developmental or environmental regulator during seed dormancy and germination.

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# Index

- 14-3-3 proteins 24, 154  
ABA2 3, 5–6, 8–11  
ABA3 3, 6, 10–11  
ABAP1 16, 25  
ABH1 18  
ABI5 binding protein (AFP) 13–14  
abiotic stresses 1, 9–12, 16–21, 23–5,  
86, 100, 108–11, 156–7, 185–8, 192,  
194, 206, 208, 216, 234, 238–41,  
245–6, 295–7  
abscisic acid (ABA) 1–35, 136–8, 201,  
206–8, 210–11, 213, 216–17, 296,  
303, 311–12, 314–19, 323–4,  
327–31  
ABA 8'-hydroxylase (CYP707A) 7, 5,  
9–11, 317, 324  
ABA-activated protein kinase, AAPK  
20–1  
ABA-binding proteins 15–16  
ABA biosynthesis 2–6, 323  
ABA deactivation 6, 7, 317, 323, 330  
ABA-deficient mutants 1, 6–12, 211,  
318  
ABA glucosyl transferase 7  
ABA-hypersensitive mutants, *era3*  
330  
ABA-insensitive mutants (*abi1*, *abi2*,  
*abi3*, *abi4* and *abi5*) 13, 17, 24, 296,  
314–16, 319, 328–30  
ABA receptor 1, 15–16, 25, 296, 328  
ABA-responsive elements (ABREs)  
13–14, 17, 167, 314–17, 319  
ABA response promoter complexes  
(ABRCs) 329  
ABA signal transduction 12–24  
ABSCISIC ACID-INSENSITIVE  
ABI1, ABI2 14, 19, 21, 24, 25  
ABI3 12–14, 19, 314–17, 319  
ABI4 13, 17, 316–17  
ABI5 12–14, 315–17, 319, 328, 329  
abscisic aldehyde oxidase (AAO3) 3, 4, 6,  
8–11  
abscisin 2  
ACC-RELATED LONG HYPOCOTYL 1  
(ALH1) 296  
*acx1* 199, 200, 211  
acyl-CoA oxidase (ACX) 195, 200  
acyl-CoA synthase 195  
AGAMOUS (AG) 153, 298  
AGAMOUS-like 15 (AGL15) 153, 318  
AIP2 (E3 ligase) 13  
AKIP (RNA-binding protein) 21, 23  
aleurone cells 15–16, 19, 147, 159, 166,  
168, 170, 277, 324–9  
allene oxide synthases (13-AOSs) 191–2  
alternative oxidase (AOX) 235, 246  
 $\alpha$ -amanitin 313  
amidase 42  
1-aminocyclopropane-1-carboxylic acid  
(ACC) 126–7, 130, 131, 134, 197,  
202, 304  
ACC conjugation (malonylation,  
glutamylation) 126  
ACC oxidase (ACO) 126–7, 130–1  
ACC synthase (ACS) 126–30, 304  
2-aminoindan-2-phosphonic acid (AIP)  
230, 234  
 $\alpha$ -amylase 147, 159, 166–8, 170, 277,  
319, 326–9  
anther development 109, 168, 172, 193,  
199, 200, 215, 294, 297–8, 300  
anthocyanin synthesis 298  
anthranilatephosphoribosyl transferase 38  
*Arabidopsis* SKP1-like (ASK) 53, 163,  
203–4  
ARGINASE 211  
arginine decarboxylase 301  
ascorbate peroxidase (APX) 238  
autonomous flowering pathway 16, 25,  
293, 297

- AUX1/LAX influx carriers 263–5, 267–9  
 auxin 12, 23, 25, 37–66, 97, 129, 137, 138, 154, 176, 195, 198–9, 202, 204–6, 213–5, 295–303  
   auxin distribution and transport 257–69  
   auxin efflux carriers 261–3  
   auxin homeostasis 257–8, 260  
   auxin influx carriers 263, 265, 268, 269  
   auxin receptor 55–8  
   auxin signal transduction 46–57, 202, 204, 214  
 AUXIN BINDING PROTEIN 1 (ABP1) 56, 58  
 Auxin Resistant (AXR)  
   *AXR1* 53, 56–7, 201–3, 205, 214  
   *AXR2/IAA7* 49, 214  
   *AXR6* 52  
*AUXIN RESISTANT 6, AXR6* 52  
 AUXIN RESPONSE FACTORS (ARFs) 47–53, 54, 58, 199, 215, 296, 298, 303  
 Auxin-Responsive Elements (AuxRE) 47–51, 53–5, 57–8, 296  
 AUXIN SIGNALING F-BOX (AFB) proteins, AFB1, AFB2, AFB3 57, 58  
 AUXIN/INDOLE-3-ACETIC ACID (AUX/IAAs) 46–51, 53–5, 57–8, 297, 303  
 avirulence (AVR) gene 48, 50  
  
*BAS1* 71, 75–6, 78–9, 295  
 BEL1-like proteins 153  
 benzoic acid (BA) 230–2, 234, 237  
 benzoic acid 2-hydroxylase 232  
 benzoic acid pathway 230, 234  
*BES1 INTERACTING MYC-LIKE 1 (BIM1) BIM2 and BIM3* 80–6  
*big/tir3/doc1* 267  
 biotic stress 12, 109–12, 126, 185–6, 188, 208, 234, 239  
 BNST3, BNST4 (steroid sulfur transferase) 71, 77  
 BODENLOS (BDL/IAA12) 49, 51, 57  
 brassinolide (BL) 67–71, 74–8, 279–80, 330–1  
   BL synthase (CYP852, CYP85A3) 70–1, 74  
 brassinosteroids (BRs) 12, 67–92, 137–8, 154, 206, 257, 277–83, 295, 303, 329, 331  
   BR biosynthesis 67–79  
   BR 2-hydroxylase (ROT3/CYP90C1) 70–1, 73–4  
   BR 3-oxidase (SAX1/CYP90D) 70–2  
   BR 6-oxidase (Dwarf/CYP85A/BRD1) 70–2, 74, 79, 295  
   BR 22-hydroxylase (DWF4/CYP90B1) 70–1  
   BR 23-hydroxylase (CPD/CYP90A/CBB3/DWF3/COS1 0/dpy) 70–1, 73, 75  
   BR 26-hydroxylase (BAS1/CYP734A1) 71  
   BR deactivation 75–6, 295  
   BR-deficient mutants 69, 71–8, 154, 279, 281–2, 295, 303  
   BR distribution and transport 277–83  
   BR down-regulated (BRD) genes 82–5  
   BR receptor 69, 78, 80–1, 83–4, 86, 208–9  
   BR response element (BRRE) 83  
   BR signal transduction 80–6  
   BR up-regulated (BRU) genes 82–5  
 BRASSINOSTEROID-ASSOCIATED KINASE 1 (BAK1) 80–1, 83–6  
 BRASSINOSTEROID INSENSITIVE (BRI1) 69, 78, 80–6, 330  
 BRASSINOSTEROID, LIGHT AND SUGAR 1 (BLS1) 75  
 brassinazole 72, 73, 82  
 BRASSINOZOLE-RESISTANT 1, 2 (BZR1 and BZR2) 78, 80–6  
 brefeldin A (BFA) 267, 268  
 BR INSENSITIVE 2 (BIN2) 80–6  
 BRI1 EMS SUPPRESSOR 1 (BES1) 80–6  
 BRI1 SUPPRESSOR 1 (BSU1) 80–1, 83–6  
 BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester) 237, 245  
*BX1* 39  
  
 calcineuren B-like (CBL9) 11  
 calcium 2, 11, 15, 21–3, 47

- calcium-dependent protein kinase (CDPK) 130, 304
- carbonic anhydrase (CAN) 238
- $\beta$ -carotene epoxidase (BCH) 3
- carotenoid cleavage dioxygenase (CCD) 6
- carotenoids 2–6, 9–11, 303, 314, 317
- catalase 238
- CCAAT-box 314
- CCAAT-box-binding factor 314
- cell wall oligogalacturonides (OGAs) 210–11
- cellulose synthase 200, 203
- CEV1* 200, 203, 206, 214
- CHASE domain 101, 105
- CHD3 proteins 316
- chemiosmotic hypothesis 261, 263–5
- CHIB1 2 (CH12)* 71, 76
- 3-chloro-4-hydroxyphenylacetic acid (CHPAA) 261
- 4-chloro-IAA 154, 258, 301
- chorismate 231, 233
- circadian rhythms 129, 138
- 9-*cis*-epoxycarotenoid dioxygenase (NCED) 3–6, 8–12, 317
- class I 1,3- $\beta$ -glucanase 330
- class-I KNOTTED1-like homeobox (KNOX) transcription factors 97  
*see also KNOX*
- climacteric fruits 302–4
- COMATOSE (CTS)*, *PXA1* 194–5
- CONSTANS (CO)* 214, 295, 297
- CONSTITUTIVE DISEASE RESISTANCE1 (CDR1) 244
- CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) 14, 204
- CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) 70–1, 73, 77–8, 83, 295
- CONSTITUTIVE TRIPLE RESPONSE (CTR) 130–4
- COP10 204
- COP9 signalosome (CSN) 55–6, 203–4
- copper Cu(I) 125, 131
- copper transporter (P-type ATPase, RAN1) 131
- CORONATHINE-INSENSITIVE 1 (COI1) 201–8, 211, 214–16, 242, 245
- CORONATHINE-INSENSITIVE SUPPRESSOR 1 (COS1) 200, 203–4
- coronatine 193, 202, 204, 207
- coupling element1 (CE1) 316
- CRE1/AHK4 cre1/ahk4* 101–3, 105
- cross-talk
- hormone-hormone 21, 137–8, 185, 202, 205, 207, 213–15, 217, 242, 246, 257
- light-hormone 24, 230
- sugar-hormone 105, 136
- CTR1 132–5, 240, 302, 330
- CULLIN1 (CUL1) 52, 55–6, 162, 163, 203–5
- CULLIN3 (CUL3) 128–9
- CULLIN ASSOCIATED AND NEDD8 DISSOCIATED 1 (CAND1), *cand1* 55, 56
- $\beta$ -cyanoalanine synthase 126
- cytochrome P450 monooxygenases (P450s) in ABA deactivation 5, 7, 9–11, 317, 324
- in BR biosynthesis and deactivation 71–9, 85
- in cytokinin biosynthesis 95–6
- in GA biosynthesis and deactivation 148–50
- in IAA biosynthesis 41
- in JA biosynthesis 192
- cytokinin 93–123, 129–30, 136–8, 153, 295–6
- cytokinin biosynthesis 153
- cytokinin catabolism 98–100
- cytokinin conjugation 97–8
- cytokinin oxidase/dehydrogenase (CKX) 95, 98–100
- cytokinin receptor 101–6, 108
- cytokinin receptor mutants 104
- CYTOKININ INSENSITIVE (CIN) 129
- D18* 152
- D35* 152
- DARK-INDUCED DWF-LIKE PROTEIN 1 (DDWF1)* 71, 74

- DDF1* 156–7  
*def1* 211  
 dehydration responsive element binding (DREB) protein 156  
 DELLA proteins 138, 154, 157–65, 167–9, 173–5, 322, 328–9  
   *see also* GAI, RGA, RGL1, 2, 3, SLN1, SLR1  
 1-deoxy-D-xylulase-5-phosphate (DXP) 3, 94  
 1-deoxy-D-xylulase-5-phosphate synthase (DXS) 3–4  
 derubylation 128  
 desiccation tolerance 1, 8–9, 311, 313, 319  
*DETIOLATED 2 (DET2) det2* 69–73, 78–9, 295, 303, 330  
 2,4-dichlorophenoxyacetic acid (2,4-D) 39, 261, 265  
 dihydrophaseic acid (DPA) 5, 7, 323  
 dihydrozeatin (DHZ) 95  
 dimethylallyl diphosphate (DMAPP) 2–4, 94–6  
 $\alpha$ -dioxxygenases ( $\alpha$ -DOX) 187–8  
*DIR1* 235, 244  
 divinyl ether synthases (DESSs) 188, 191–2  
*DR12* 303  
*DUMPY (DPY)* 71, 75  
*DWARF d' (CYP85A1)* 71–2, 74, 281, 282  
*DWARF 11 (D11) (CYP724B1)* 75  
*DWARF1 (D1)* 158, 165  
*DWARF4 (DWF4) (CYP90B1)* 71–3, 83, 295  
*DWF1* 295  
  
*E1-LIKE CONJUGATING ENZYME RELATED 1 (ECR1)* 53, 56  
*E3* ubiquitin ligase 13, 128, 129, 166  
   *see also* SCF *E3* ubiquitin ligase  
*Early heading date 1 (Edh1)* 114  
 E-box sequence motif 83  
*EDR1* 235, 240  
 EIN3-binding F-box protein (EBF) 135–6  
 EIN-like (EIL) 135–6, 302, 303  
*ELONGATED UPPERMOST INTERNODE (EUI)* 149–50  
*Em* promoter 328  
 endo-b-mannanase 325  
  
*ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)* 216, 234–5, 244–5  
*enhanced-ethylene-response (eer)* mutants 131  
*ent-copalyl diphosphate synthase (CPS)* 148, 151–2  
*ent-kaurene* 148, 151–2, 170–4, 327  
*ent-kaurene oxidase (KO)* 148–9, 151–2, 154, 174, 317  
*ent-kaurene synthase (KS)* 151–2, 274  
*ent-kaurenoic acid hydroxylase (KAO)* 148–9, 151–2  
 epoxyalcohol synthases (EASs) 188, 191–2  
 ethylene 8, 12, 49, 50, 108–12, 125–45, 185, 197, 201–2, 204–8, 210, 213, 217, 229, 240, 242, 244–6, 257, 295–6, 302–4, 329–30  
   ethylene biosynthesis 126–31, 303  
   ethylene signal transduction 131–7  
   ethylene receptors 131–5, 302–3, 330  
 ETHYLENE INSENSITIVE (EIN) 130, 132, 134–6  
   EIN2 *ein2* 12, 134–6, 302, 330  
   EIN3 *ein3* 135–6, 201, 302–3  
   EIN4 131–2, 135  
 ETHYLENE OVERPRODUCTION (ETO) *eto* 128–31  
 ETHYLENE RESISTANT (ETR, ERS) 130–5, 302  
 ETHYLENE RESPONSE FACTOR (ERF) 17, 21, 135–6, 138, 202, 208, 216, 302–3  
 ETHYLENE-RESPONSIVE ELEMENT-BINDING PROTEIN (EREBP) 135–6  
*ETTIN (ETT)* 49, 298–9, 301  
 expansins 166–7, 325  
  
 F-box protein (*see also* COI1, TIR1, SLY, SNE) 52, 54–5, 57, 135–6, 158, 162–3, 174, 200–4, 216  
*FCA, fca* 1, 16, 25, 296–7  
 feedback regulation  
   negative 10–11, 15, 47, 50, 53–4, 69, 73, 77–8, 80, 82–3, 97, 101–2, 107, 115–16, 129, 148, 154, 159, 169–70, 174–5, 295

- positive 11, 18, 185, 202, 209, 235, 238
- feedforward regulation 11, 78, 130, 156, 169, 174
- flavin monooxygenase 41, 298
- flavonoids (as PAT inhibitors) 261, 267
- FLC* 296
- floral homoeotic genes 297–8
- fluridone (ABA biosynthetic inhibitor) 323–4
- FPP1* 294
- FRIGIDA (FRI)* 296
- fruit development and ripening 37, 74, 125, 130, 147, 191, 270, 299–304
- fry2* 18
- FT* 295, 297
- FUSCA3 (FUS3), *fus3* 12–15, 153, 314–16, 318–19, 331
- GA response elements (GAREs) 167
- GAI, *gai-1* 97, 158, 160–1, 163, 169, 173, 294–5, 322
- GAMYB 166–8, 294, 328–9
- gar2-1*, *sly1-d* 163
- GARP motif 107
- G-box 316
- GCC-box promoter elements 303
- gentisic acid 235–6
- geranylgeranyl diphosphate (GGPP) 2, 3, 148
- geranylgeranyl diphosphate synthase (GGPS) 3
- GH3* 43, 45–7, 50, 58
- gibberellin (GA) 12, 14–15, 21, 97, 137, 147–84, 293–301, 311–12, 315–31
  - GA 13-hydroxylase 149–50
  - GA 2-oxidase (GA2ox) 152–6, 169, 172, 174, 273, 301, 317–18, 320–2
  - GA 20-oxidase (GA20ox) 97, 149, 151–6, 169–74, 273, 276, 295, 301, 318, 320
  - GA 3-oxidase (GA3ox) 14, 97, 149–50, 152–5, 169–74, 273, 276, 295, 301, 315, 318–22, 325–6
- GA biosynthesis 137, 147–57, 159, 164, 170–5, 270–4, 294–6, 300–1, 315–21, 324–7
- GA conjugation 150
- GA deactivation 97, 150, 152–4, 156, 273, 277, 318–22
- GA distribution and transport 269–77
- GA-deficient mutants 147, 160, 167, 169, 173, 270–1, 274, 275, 294, 297–8, 322–5, 324–5, 330
- GA receptor 147–8, 157–9, 164–5, 174–5, 328
- GA response mutants 159, 161–2, 165, 322, 328
- GA signal transduction 97, 138, 157–70, 277, 294, 298, 322–3, 327–9
- gibberellin-dependent flowering pathway 293
- GIBBERELLIN-INSENSITIVE DWARF 1 (GID1)* *gid1* 157–9, 164–5, 175, 328
- GIBBERELLIN-INSENSITIVE DWARF 2 (GID2)* 157–8, 162–4
- GIGANTEA, GI* 294
- glucose 7, 11, 12, 13, 19, 44–5, 185, 194, 198–9, 231, 236
- glucose signaling 136–8
- glutamate receptor (AtGLR1) 12
- GNOM 267
- GPA1* 23, 158
- G-proteins 22, 23, 25, 158, 165, 267, 331
- G-protein coupled receptor (CR1), *gcr1* 23
- grafting 270–2, 274, 277, 279–81
- Grain number 1a (Gn1a)* 100
- GRAS proteins 158, 160–2, 322
- gravitropism 262, 264–6, 268–9
- guard cells 1, 2, 10, 15–16, 19–25
- H<sup>+</sup>-ATPases 22, 24
- heterotrimeric G-proteins *see* G-proteins
- high-affinity sulfate transporter genes (*SULTR1*) 104
- HIMI,2,3* 166
- histidine kinase receptors (HK, AHK) 101–6, 108, 116, 131–2
- histidine phospho-transfer protein (AHP) 101–3, 105, 106, 108–9, 112, 116
- histone deacetylase (HDA) 203–4
- homeodomain proteins, AtHB6 19, 21



- HOOKLESS HLS* 50, 135  
*HOOKLESS SUPPRESSOR 1, HSSI/ARF2* 50  
 Hormone Sensitive Lipase (HSL) family 158–9  
 hydrogen peroxide 206, 210–11, 238, 240  
 hydroperoxy lyases (13-HPLs) 191–2  
 hydroxymethylbutenyl diphosphate (HMBPP) 95, 96  
 hypersensitive response (HR) 191, 205–6, 235, 239–40, 242  
*HYPERSENSITIVE TO ABCISIC ACID AND AUXIN, sax1* 71–2  
*HYPONASTIC LEAVES 1, HYL1* 18, 296  
  
 IAA oxidase 46  
 IAA-L-aspartate hydrolase 44  
 IAP1 43  
 IAR3 44  
 IFL 266  
 IGL 39  
 ILL2 44  
 ILR1 44  
 INA (2, 6-dichloroisonicotinic acid) 237–8, 241  
 indole 39, 40  
 Indole-3-acetic acid (IAA) 37–47, 50, 57, 58, 154, 258–62, 265–6, 268, 283  
   IAA biosynthesis 37–42, 258–9, 298, 300  
   IAA conjugates 42–6, 258  
   IAA conjugate hydrolysis 43–5  
   IAA oxidative degradation 46, 258  
 indole-3-acetyl transferase 45  
 indole-3-glycerolphosphate (IGP) synthase 39  
*iso* chorismate 231, 233–4  
*iso* chorismate pathway 233–4  
*iso* chorismate pyruvate lyase (IPL) 233  
*iso* chorismate synthase (ICS) 233–4  
*iso* pentenyl diphosphate (IPP) 2, 3, 4, 94, 148  
*iso* pentenyl diphosphate transferase (IPT) 94–7, 99, 153  
*iso* pentenyladenine (iP) 94–6, 103  
*iso* pentenylmonophosphate (iPMP)-dependent pathway 96  
  
*iso* pentenylmonophosphate (iPMP)-independent pathway 95–6  
  
*jail* 201–2, 207, 211, 215  
 Jasmonic acid (JA) 77, 136, 138, 185–229, 235, 240, 242, 245–6  
   JA-accumulating mutants 206, 214  
   JA biosynthesis 192–7  
   JA-insensitive mutants 193, 206, 214  
   JA metabolites 197–9  
   JA receptor 205, 208  
   JA signal transduction 200–5  
 JA-amino synthase 47  
 JA-conjugate synthase (JAR1) 47, 197–8, 201–2, 206  
 JA-specific methyl transferase 197–8  
 JIN1, *jin1* 201–2, 208, 214  
  
 KNOX 97, 153  
  
 L-3-ketoacyl CoA thiolase (KAT) 195  
 late embryogenesis abundant (LEA) proteins 17, 312–13  
*LEAFY COTYLEDON 1 (LEC1) lec1* 314–16  
*LEAFY COTYLEDON 2 (LEC2) lec2* 13, 14, 314–16, 318–19, 331  
*LEAFY COTYLEDON 3 (LEC3)* 153  
*LEAFY, LFY* 294–5  
 leucine rich repeat receptor-like kinase (LRR-RLK) 80, 83  
 leucine-rich repeat receptor kinase (LRR kinase) 201, 203  
 light 37, 50, 75–6, 78, 79, 129, 138, 155–6, 168, 293, 295, 297, 302, 311, 319–23, 325, 330  
   blue 23–4, 79, 155  
   far-red 79, 155, 320–3  
   red 116, 155, 320–1, 323  
   UV 211, 234, 297  
 lipid peroxidation 187, 191  
 lipoxygenase (LOX) pathway 185, 188–92, 205, 212, 217  
 lipoxygenases 185, 188, 190, 193  
   type1-LOXs 190  
   type2-LOXs 190  
*LK* 71–2

- lkb* 279–81  
 lumazine synthase 200, 203–4  
 lycopene synthase (LCYB) 3
- MADS box transcription factor 153, 318  
 MATE transporter 234  
 MDR/PGP 263–6, 269  
 MEMBRANE STEROL BINDING  
   PROTEIN 1 (MSBP1) 83–4, 86  
 MERISTEM LAYER 1 (AtML1) 15, 318  
 methionine cycle 126–7  
 methyl jasmonate (MeJA) 185, 189, 198, 208, 212, 214, 229, 237, 244  
 methylerythritol phosphate (MEP)  
   pathway 2–4, 94, 96, 148  
 methylsalicylate (MeSA) 212, 231, 235–8, 243  
   MeSA esterase 237  
   MeSA-2-O- $\beta$ -D-glucoside 231, 237  
 mevalonic acid (MVA) 2, 3, 94–6, 148  
 micro RNAs (miRNAs) 17, 18, 51, 168, 294  
 microarrays 17, 23, 50, 83, 116, 130, 136–8, 147, 167, 172, 215–16, 244, 303, 313, 316, 325  
 mitogen-activated protein kinase (MAPK) 130–1, 134, 135, 202, 217, 235, 239–40, 302  
   MAPK cascade 14, 134–5, 239–40  
   MPK4 201–2, 207  
   wound-induced MAPK (WIPK) 239  
 MMK 134  
 moco sulfuryase (ABA3, FLACCA) 3, 6, 10–11  
*MONOPTEROS (MP)/ARF5* 50–1, 298  
 multifunctional protein (MFP) 195, 200  
 MYB transcription factors, *atmyb60*, *atmyb61* 17, 24, 239
- N tobacco resistance gene 239  
*NahG* gene 237, 243, 245  
 naphthalene-1-acetic acid (NAA) 261  
 1-naphthoxyacetic acid (1-NOA) 261  
*NEVER-RIPE (NR)* 302, 304  
 nitrate 8, 97, 323  
 nitric oxide (NO) 210–11  
 nitrilase 42
- N*-1-naphthylphthalamic acid (NPA) 50, 261, 265–9, 298  
*NON-expressor of PR1 (NPR1)* 207, 235, 240–3, 245–6  
*NON-PHOTOTROPIC HYPOCOTYL 4 (NPH4) ARF7* 50  
 NTH15 153  
   *see also KNOX*
- octadecanoid pathway 211, 213  
 O-linked N-acetylglucosamine  
   transferases 164–5  
   *see also SPINDLY*  
 OPDA reductases (OPR3) 188, 1946, 199, 200, 202, 211, 215  
 orange pericarp mutants, *orp1*, *orp2* 38, 40  
 ORE9 200, 203, 216  
 OST1 kinase 17, 20, 24  
 OXII 240  
 2-oxoglutarate-dependent dioxygenases (2ODDs) 148–51, 169  
 12-oxophytodienoic acid (OPDA) 185, 188–9, 193–7, 199, 202, 205, 207, 211, 229  
   *see also* jasmonic acid  
 oxylipins 185–228  
 ozone 126, 201, 206, 208, 234
- paclobutrazol (PAC) 161, 297, 300, 327  
 parthenocarpy 299, 300  
*pat-1* mutant 300  
 pathogens, response to 93, 126, 136, 138, 185–8, 192, 198–202, 204–8, 216, 229, 232–46, 277, 297, 302, 304  
 pathogen-related (PR) genes, proteins 202, 207, 237, 282  
 peroxisomes 194–6  
 peroxygenases (POXs) 188, 191–2  
 phaseic acid (PA) 5–7, 10, 11, 323  
 phenylalanine 230–2  
 phenylalanine-ammonia lyase (PAL) 230, 234, 239  
 phenylpropanoid pathway 230–2, 234  
 phosphatidic acid 19  
 phospholipase D 16  
 phospho-relay system 101–2, 130–1  
 PHOT1, 2 23–4

- photomorphogenesis 67, 69, 73, 76, 79, 295  
 photoperiod-dependent flowering  
   pathway 114, 156, 171, 214, 293–4, 297  
*PHOTOPERIOD-RESPONSIVE 1*  
   (*PHORI*) 165–6  
 phototropism 137, 260, 266  
 PHYB4 ACTIVATION-TAGGED  
   SUPPRESSOR 1 (see BAS1)  
 phyllotaxy 117  
 PHYTOALEXIN DEFICIENT4 (*PAD4*)  
   207, 216, 234–5, 244, 245  
 phytoalexins 212  
 phytochrome 116, 155–6, 194, 320–2  
   phytochrome A (*PHYA*) 155, 320–1  
   phytochrome B (*PHYB*) 116, 155–6, 320  
   phytochrome E (*PHYE*) 320–1  
 phytoene synthase (*PSY*) 2–3  
*PICKLE, PKL* 316  
*PID* 267  
*PIL5* 155, 321  
 PIN-FORMED 1 (*PIN1*), auxin efflux  
   carrier 263–9, 298  
*PINOID* kinase 267–8  
*PKABA1* (ABA-inducible Ser/Thr  
   protein kinase) 21, 328–9  
 plant pathogen interactions 187–8, 206–7  
 polar auxin transport (*PAT*) 260–9, 298  
 pollen development 8, 168, 186, 199, 297–9  
 pollen release 215, 297  
 polyamines 126, 299, 301–2  
 polyunsaturated fatty acids (*PUFAs*) 185, 188–91  
 potassium ( $K^+$ ) 22–3, 108, 111  
 precocious germination 14, 313, 317  
 programmed cell death (*PCD*) 108–12, 206–7, 235  
 prosystemin 200–1, 208–9  
 26S proteasome-mediated protein  
   degradation 13–14, 47, 49, 52–5, 83–4, 128, 135–7, 157, 162, 164, 166, 175, 202–3, 242, 322  
 proteasome inhibitors *MG132* 82  
 protein phosphatases 2C (*PP2C*) 14, 18–19, 22–5  
 proteinase inhibitors (*PINs*) 198, 208–12, 215  
 quantitative trait loci (*QTL*) 100  
 radicle emergence 324–5  
*RBX1* 52, 53, 55, 162, 203–4  
*rcd1* 201, 206  
 reactive oxygen species (*ROS*) 22, 206, 210, 235, 238, 240, 246  
 receptor-like protein kinases (*RPKs*),  
   *rpkl* 16  
 redox status 235, 238, 241–2  
 REGULATORY PARTICLE  
   NONATPASE 10 (*RPN10*) 14  
 RELATED TO UBIQUITIN (*RUB*) 53, 55–6, 203–5  
 resinosis 212  
 resistance (*R*) gene 205, 208, 235  
 response regulators (*ARRs*) 101–2, 104, 106–7, 109, 110–16, 130–2, 134  
   A-type *ARRs* 101–2, 104, 106–7, 110–16  
   B-type *ARRs* 101–2, 106–7, 109–10, 112–14  
*RESPONSIVE TO ANTAGONIST (RAN)*  
   131–2, 135  
*RGA* 97, 138, 158, 160–3, 173, 294–5, 298, 322  
*rga-β17* (*RGA* gain-of-function mutation)  
   161, 169  
*RGL1*, 2, 3 158, 160, 161, 163, 298, 322  
 RNA-binding protein 1, 18, 21, 23, 25  
 root elongation 8, 50, 103, 105, 138, 186, 261, 265  
 root elongation zone 109, 111, 174, 261–2  
 root growth 19, 50–1, 80, 93, 100, 137–8, 173, 185–6, 200–2, 205, 213–14, 259, 268, 330  
 root-to-shoot communication 97, 117  
*ROTUNDIFOLIA 3 (ROT3)* 71, 73, 77, 295  
*RPD3a (HDA)* 204  
*RR* domain 112–13

- RSG (repression of stem growth) 153–4
- RUB CONJUGATING ENZYME (RCE1) 53, 56, 128, 130
- rubylation (RUB conjugation) 53, 55–6, 128, 203–4
- RY repeat elements 12–14, 314–16, 319
- salicylic acid (SA) 77, 138, 202, 205–8, 210–12, 216, 217, 229–55, 295, 297, 304
- SA biosynthesis 230–5
- SA carboxymethyltransferase 237, 243
- SA conjugates 231, 236
- SA glucosyltransferase (SAGT) 236
- SA hydroxylase 236
- SA metabolism 235–7
- SA signal transduction 237–43
- salicylate synthase 233
- SA-binding protein (SABP) 235, 237–8, 243, 244
- SAD1 11, 18
- SA-deficiency 297
- S-adenosyl-methionine (AdoMet) 126–7, 129
- S-adenosyl-methionine synthetase (SAM synthetase) 126–7
- SA-induced protein kinase (SIPK) 239–40
- SCF E3 ubiquitin ligase 157, 158, 162–4, 175, 202–5, 214
- SCF<sup>COH</sup> 203–5
- SCF<sup>GID2</sup> 157, 163–4
- SCF<sup>SLY1</sup> 163
- SCF<sup>TIR1</sup> 51–7
- SDR1 6
- SECRET AGENT (SEC)* 165
- seed development 8–9, 12–13, 40, 42, 147, 270, 311–38
- seed dormancy 1, 4, 9, 19, 311–13, 317, 319, 321, 323–4, 327, 330–1
- seed germination 9, 16, 17, 21, 42, 104, 147, 154, 155, 161, 167, 186, 214, 311–38
- SEMI DWARF1 (SD1)* 152
- see also* GA 20-oxidase
- senescence 16, 37, 50, 69, 93, 104, 126, 130, 136, 185–6, 200, 203–4, 213, 215–16, 240, 301–2
- SGT1b 55, 201–3, 205
- shoot apical meristem (SAM) 97, 113, 115, 138, 258, 276–7, 293, 297
- sieve elements 221
- silver Ag(I) 125
- SIMK 134
- SKP1-related proteins 52, 52, 55, 162–3, 203–4
- OsSKPs 163
- SLEEPY 1 (SLY1) 158, 162–3, 173
- see also* SCF<sup>SLY1</sup>
- SLENDER1 (SLR1), 157–8, 160–1, 163–4, 167, 170–2
- SLN1 161, 167, 328–9
- SLR1-like-1, 2 160
- SMALL AUXIN-UP RNAs (SAURs) 46, 47, 58
- SNEEZY (SNE) 158, 162
- SOB7 71, 76, 295
- SPATULA (SPT) 155, 298–9, 301
- Sph element 314
- sphingosine-1-phosphate 23
- SPINDLY (SPY) 158, 164–5, 294, 295
- spr2* 199, 200, 211
- see also* w-7-fatty acid desaturase
- steroid 5 $\alpha$ -reductase 69–72
- steroid sulphotransferase (SOT) 71, 77
- sterol binding proteins (SBPs) 83–4
- stomatal-closure 1, 8, 15–16, 18–24, 229
- stratification 155, 321, 323, 330
- Sucrose Non-Fermenting (SNF) 1, SNF1 19, 20
- Sucrose non-fermenting Related Kinases, SnRKs 19–21, 25
- sugar signaling 12, 13
- sulphur acquisition 104–5
- SUPERROOT 2 (SUR2)/CYP83B1* 41, 50
- suppressor of NPR1 inducible (sni1)* 235, 243
- systemic acquired resistance (SAR) 229, 237, 242–6
- systemin 208–11, 244
- systemin receptor 208–11

- temperature 86, 155–6, 168, 185, 214,  
293, 297, 302, 311, 319–23
- tendril coiling 198, 213
- TGA transcription factors 235, 241–2
- thermo-inhibition 323
- thermoperiodism 156
- TIR1 15, 51–8, 204–5  
*see also* SCF<sup>TIR1</sup>
- tobacco mosaic virus (TMV) 229, 232,  
236–9, 243
- tomato ripening mutants 304  
*see also* NEVER-RIPE
- TRAB1 315, 319
- trans-acting short-interfering RNAs 51
- trans-cinnamic acid 230–2
- trans*-zeatin secretion (tzs) 96
- triadimefon 72
- trichomes 14, 97, 100, 110, 186, 201–2,  
237, 314–15
- 2,3,5-triiodobenzoic acid (TIBA) 261,  
266, 267
- triple response 112, 125, 126, 129–30, 136
- tryptophan 37–41, 206
- tryptophan synthase 38–40
- tt4* 267, 269
- tuberization (tuberisation) 166, 191, 213,  
273
- tuberonic acid 198, 214
- two-component regulators 101, 105–6,  
115, 130
- ubiquitination 13, 52–5, 57–8, 128–9,  
135–6, 157, 162, 164, 175, 203–5,  
242, 322
- UDP-glycosyltransferase 7, 71, 77, 79,  
95, 98, 236
- UFD2 homology domain (U-box) 166
- VIVIPAROUS 1* (*VPI*) 12–14, 314–16,  
319, 328–9
- vivipary 9, 314
- volatile organic compounds (VOCs)  
212–13
- VP14 4, 6
- w-7-fatty acid desaturase 200–1
- Whirly (Why) transcription factors 235,  
243
- wol* 105, 116
- wound-inducible polygalacturonase  
211
- wound response 200–1, 207–11
- WRKY transcription factors 207, 216,  
235, 239–42, 245
- WRKY70 207
- WW domain 16
- xyloglucan endotransglycosylase (XET)  
166–7, 325–6
- Yang cycle 126–7
- YUCCA* 41, 50
- Zeatin *cis* (cZ) 95
- Zeatin *trans* (tZ) 94–6, 103
- zeatin O-glycoside transferase 95, 98
- zeaxanthin epoxidase (ZEP) 3, 4, 9–12